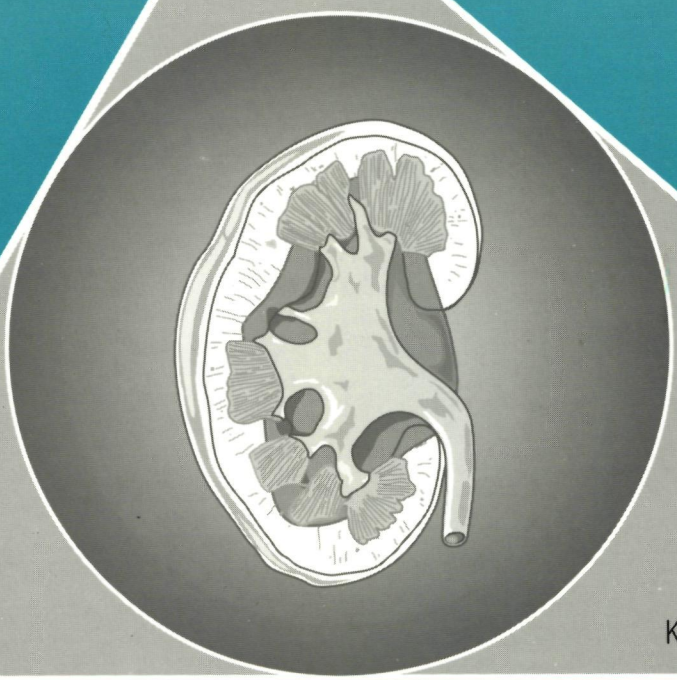
Anti-nucleosome auto-antibodies in systemic lupus erythematosus



Kees Kramers

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Aan Yvonne, Bart en Tim
Aan mijn ouders

CONTENTS

CHAPTER 1

Introduction.

Systemic lupus erythematosus (SLE) is an auto-immune disease which may affect any organ of the body. The course of the disease is unpredictable and is characterized by exacerbations and remissions (1). Nephritis is one of the most serious manifestations of the disease, and is found in about half of the patients. In 20% of the cases nephritis leads to end stage renal disease (2).

The diagnosis of SLE is based on a combination of disease manifestations and serological findings (1). In SLE a wide variety of auto-antibodies can be found. Especially antibodies against nuclear antigens are formed, but also antibodies directed against other auto-antigens for instance cytoplasmic antigens, cell membrane determinants, immunoglobulins and clotting factors (1). A major group of auto-antigens recognized by anti-nuclear antibodies are located within chromatin. Chromatin is the compact fiber in which the eukaryotic DNA is packed. In inactive chromatin, DNA is complexed to histones forming nucleosomes. In a nucleosome four pairs of the histone proteins H2A, H2B, H3 and H4 form the histone octamer. Two superhelical turns of 146 bp of DNA are wound around this octamer. Histone H1 and a linker stretch of 60 bp of DNA connect neighboring nucleosomes like beads on a string (3). In active chromatin, DNA is unfolded from the nucleosomes and gene transcription and DNA replication take place. As outlined in the table in various auto-immune diseases antibodies to nucleosomal antigens, transcriptional complexes and DNA replication/repair complexes can be found. A selection of these anti-chromatin antibodies and their association with autoimmune diseases is given in the table (next page).

Many of the nuclear antigens which serve as a target in auto-immune diseases, are complexes of proteins and nucleic acids which are involved in distinct nuclear functions. Often a set of auto-antibodies against the individual components of a complex tends to be produced tandemly. For instance the nucleosome is a frequent auto-immune target in SLE, yielding antibodies to DNA, histones and nucleosomes. This phenomenon is also found with other autoantibodies, e.g. those against small nuclear ribonucleoprotein (snRNP) autoantigens such as U1, U2, U4-6 and U5 snRNPs, where antibodies may be directed against the U-RNA and the various proteins of the particle (15).

Amongst the anti-nuclear antibodies found in SLE, those directed against double stranded DNA and against the Sm protein are specific for the disease (16). Auto-antibodies have been held responsible for many of the disease manifestations and there is evidence that anti-dsDNA antibodies are pathogenic for the development of nephritis. Firstly, a rise in titer of anti-dsDNA antibodies is found preceding renal

Anti chromatin antibodies and their relation with disease

auto-antigen	disease association	ref
nucleosomal antigens		
DNA	SLE	(1)
histones	SLE, DIL[1], RA[2], Juvenile RA	(4-6)
nucleosomes	SLE, DIL, scleroderma	(7-9)
transcription complexes		
RNA polymerase I	Scleroderma	(10)
RNA polymerase II	Scleroderma	(11)
RNA polymerase III	Scleroderma	(12)
Ku antigen	SLE, Scleroderma	(13)
DNA replication/repair complexes		
PCNA[3]	SLE	(14)

[1]DIL = drug induced lupus, [2]RA = rheumatoid arthritis, [3]PCNA = proliferating cell nuclear antigen.

exacerbations of lupus (17,18), and secondly in renal eluates of patients with lupus nephritis an enrichment for anti-dsDNA antibodies has been detected (19,20). About 10 years ago we postulated that certain anti-dsDNA antibodies are able to cross react with heparan sulfate (HS) in the glomerular basement membrane (GBM) and that this event leads to nephritis (21). HS is the negatively charged glycosaminoglycan side chain of heparan sulfate proteoglycan (HSPG). It is an intrinsic constituent of the glomerular basement membrane (GBM) and responsible for the majority of the anionic sites in the GBM (22-24). These anionic sites are the major determinants for the charge dependent permeability of the GBM. Consequently, loss of these anionic sites leads to albuminuria (25-29), while antibodies to HS induce instantly an acute selective albuminuria after intravenous injection in rats (30).

Besides the reactivity of monoclonal anti-DNA antibodies with HS (21), we found anti-HS reactivity in sera of lupus patients in association with renal disease (31,32). These data are presented in chapter 3. However, in later studies we discovered that

this cross reactivity of anti-dsDNA antibodies with HS was a feature of antibodies complexed to DNA and histones (nucleosomes) (33). In these immune complexes the histone part of the nucleosome, which is strongly positively charged, interacts with HS. In a rat kidney perfusion model subsequent perfusion of histones, DNA and anti-DNA led to binding of antibodies to the GBM, whereas after perfusion of DNA and anti-DNA without histones no GBM binding was observed (34). The involvement of histones in lupus nephritis is discussed in chapter 2.

When we studied the cross reactivity of monoclonal anti-dsDNA antibodies with HS, we found for some antibodies that not only HS reactivity was due to antibodies complexed to nucleosomes, but also anti-dsDNA reactivity. In pure non-complexed form these antibodies appeared to be anti-nucleosome antibodies (35). These findings are described in chapter 4. In recent years much research has focused on anti-nucleosome antibodies. These antibodies exclusively react with the intact nucleosome and not with its components histones and DNA. Maybe it is better to speak of nucleosome-specific antibodies, for anti-DNA and anti-histone antibodies will also bind to the nucleosome.

Antibodies to nucleosomes were in fact the first auto-antibodies described in association with SLE. The morphological characteristics of the first serological marker for SLE 'the LE cell phenomenon' (36) evolve after opsonization of 'LE cell factors' complexed to DNA and histones. Later on, the 'LE cell factors' were identified as auto-antibodies and it was shown that nucleosomes were able to inhibit the formation of the 'LE cell phenomenon' in contrast to free dsDNA or histones (37). Therefore, it appears that the 'LE cell phenomenon' is related to anti-nucleosome auto-antibodies.

In recent years monoclonal anti-nucleosome antibodies have been described both in human (38) and murine lupus (39,40). Anti-nucleosome antibodies have been found to develop early in life in lupus mice, before the occurrence of anti-dsDNA and anti-histone antibodies (41,42). In humans, the prevalence of anti-nucleosome antibodies in SLE is very high (7,43), but until now in only one study anti-dsDNA and anti-histone antibodies were preadsorbed before measuring anti-nucleosome reactivity. In this study the prevalence of these antibodies was 84% (44). Also in drug induced lupus anti-nucleosome antibodies have been detected (8).

There is now generally agreement that the auto-antibody response in lupus is antigen driven since somatic mutation and affinity maturation occurs and T-cell help is required (41,45). Very interestingly, recently it was found that pathogenic T-helper cells in SNF1 lupus mice are specific for nucleosomes and also able to induce anti-

dsDNA and anti-histone antibodies (46). So, the antigen driving the autoimmune response in SLE might very well be the nucleosome. This idea is sustained by the recent finding that the antibody response in lupus is directed against antigens (nucleosomes and other nuclear antigens) which can be found in blebs, appearing on apoptotic cells (47).
Until now little is known about anti-nucleosome antibodies. For instance it is not known what epitopes are recognized on the nucleosomes and in which way the interaction with both DNA and histones takes place. Also the pathogenic significance of these antibodies has not been studied yet.

The aim of this study was to evaluate the role of anti-nucleosome antibodies in systemic lupus erythematosus. The binding mechanism of antibodies complexed to nucleosomes in the GBM was studied in a rat kidney perfusion model (chapter 4). Renal biopsies of human lupus nephritis were stained for histones and nucleosomes in glomerular deposits. The appearance of histones and nucleosomes was associated with histopathological findings and with HS staining in the GBM (chapter 5). When hybridomas producing these anti-nucleosome antibodies were inoculated intraperitoneally in naive mice, not only glomerular localization was observed but also binding to cell nuclei (*in vivo* ANA). Therefore the possible role of anti-nucleosome antibodies complexed to nucleosomes and the mechanism of *in-vivo* ANA was analyzed (chapter 6). The specificity of anti-nucleosome antibodies on different subnucleosome structures was assessed and binding to linear histone epitopes was studied (chapter 7). Based on the observation that nucleosome specific T-helper cells could drive the anti-dsDNA response (46), we analyzed whether true anti-dsDNA antibodies also recognized nucleosomal proteins (chapter 8). And lastly, since apoptosis is in all likelihood the main source for nucleosomes in the circulation and derangement of apoptosis has been found in several lupus mouse strains (48,49), the role of apoptosis in lupus, both in the induction and the effector phase of the disease, is reviewed (chapter 9).

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CHAPTER 2

Histones in lupus nephritis.

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Histones in Lupus Nephritis

Key Words
Systemic lupus erythematosus
Histones
Nephritis
Anti-DNA cross-reactivity
Heparan sulphate
Nucleosome
Glomerular basement membrane

Lupus nephritis (LN) is one of the most severe complications of systemic lupus erythematosus (SLE), ultimately affecting 30–50% of patients [1]. In 15–20% of these patients it leads to end-stage renal disease [2].

Anti-DNA Antibodies and Lupus Nephritis

The pathogenesis of LN is still unclear, despite three decades of extensive research. The presence in LN of mesangial, subepithelial and subendothelial deposits of immunoglobulins and complement factors suggests an immunecomplex mechanism. Renal exacerbations are often preceded by a rise in titer of high avidity anti-dsDNA antibodies [3–5] although this rise is not observed in every exacerbation and sometimes serological activity is seen in conjunction with a stable clinical course [6]. Renal eluates contain predominantly high avidity anti-dsDNA antibodies [7, 8]. Therefore, it seems justified to assign a pathogenic role to anti-dsDNA antibodies in LN [9, 10]. These observations have led to the classical concept that anti-dsDNA antibodies and DNA form immune complexes in the circulation, which then become trapped in the glomerular basement membrane (GBM), thereby initiating glomerular inflammation. The last decade however, new hypotheses have been put forward, since definite proof for this classical concept failed to appear [10].

Cross-Reactivity of Anti-DNA

An alternative explanation for the pathogenicity of anti-DNA is based on the observation that anti-DNA may crossreact with several other molecules, as first described for cardiolipin and other negatively charged phospholipids [10–13]. A few years ago, we described the cross-reaction of anti-DNA with an intrinsic constituent of the GBM: heparan sulphate (HS) [14]. This HS is the negatively charged side chain of heparan sulphate proteoglycan (HSPG) and responsible for the majority of the anionic sites in the GBM, which are the major determinants for the charge-selective permeability of the GBM. Loss of these anionic sites leads to proteinuria. In both human and murine SLE sera we found anti-HS reactivity, which could be inhibited by DNA, while the anti-DNA binding was inhibitable with HS [14]. The potential pathogenic significance of this cross-reactivity was underlined by the finding that anti-HS reactivity was present in renal eluates of SLE patients and lupus mice [14]. Clinically, we found a correlation of anti-HS reactivity with renal disease, first in a selected cohort of SLE patients [15], but later on also in a prospective analysis of 72 consecutive SLE patients [16]. Also studies in an isolated perfused rat kidney model, in which proteinuria developed after perfusion with an anti-DNA monoclonal antibody (mAb) or purified IgG isolated from sera of patients with

lupus nephritis, pointed towards a binding of anti-DNA antibodies to intrinsic glomerular constituents [17]. Moreover, it was recently reported that cross-reactive anti-DNA antibodies predominated in renal eluates from lupus patients [18].

Histones and Cross-Reactivity

To study the cross-reactivity with HS in more detail we used a panel of anti-DNA mAb from lupus-prone mice. Initially, these studies confirmed the binding of anti-DNA to HS and the parental molecule HSPG, since 40% of the mAb bound to HS and this binding could be inhibited by DNA and vice versa [19]. However, when we purified hybridoma culture supernatants of these cross-reactive mAb under dissociating high salt conditions (3 *M* NaCl) on a protein A-Sepharose column, they completely lost their property to bind HS and HSPG. Reconstitution of the purified mAb with the protein-A column effluent restored the HS and HSPG binding in ELISA.

Subsequently, we identified histones and DNA in the protein-A column effluent [20]. Certain unpurified anti-DNA mAb also bound strongly to isolated GBM loops, while after purification the binding disappeared. Apparently, this binding occurred through anionic sites in the GBM, since preincubation of the GBM loops with cationic ferritin prevented binding and pretreatment with the HS degrading enzyme heparitinase reduced the binding considerably [19]. After preincubation of the GBM loops with histones and subsequently with DNA the purified mAb again bound in a fine granular pattern [21]. Taken together these experiments show that the presumed cross-reactivity of anti-DNA with HS is mediated via a ligand consisting of histones and DNA, i.e. nucleosome particles. Within the nucleosome the histone part is responsible for the binding to HS, HSPG and the GBM. At the same time Jacob et al. [22] came to the conclusion that the cross-reactivity of anti-DNA with a cell membrane protein (which they named lupus-associated membrane protein 'LAMP'), was also due to the same intermediate ligand. More recently, it was shown that anti-DNA mAb can also bind to endothelial cells via histones and DNA [23]. It can therefore be concluded that a number of 'cross-reactions' of anti-DNA is mediated through histones and DNA [24].

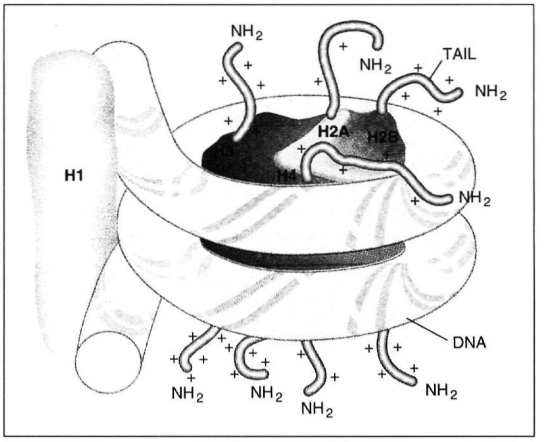

Fig. 1. The nucleosome, consisting of the histone octamere, around which 145 bp of DNA are wrapped twice. Please note that the positively charged N-terminal regions of the histones are located on the outside of the nucleosome. Histone H1 interacts with the octamere/DNA complex and bridges adjacent nucleosomes. Reproduced, with permission from Grunstein M: Histones as regulator genes. Scient Am 1992;October issue:40–47.

Histones as Nephritogenic Antigens

Histones are a set of DNA-binding proteins, which have a function in the packing of DNA molecules. Two of each of the four core histones (H2a, H2b, H3 and H4) form together an octamere around which 145-bp of DNA are wrapped twice. Histone H1 is bound to the core particle consisting of the histone octamere/DNA complex and together they form the nucleosome. The core histones are single polypeptide chains, having a molecular weight from about 11 to 15 kD. They are positively charged with a pI of about 11. The basic residues are clustered in the N-terminal part of the molecules and these positively charged regions are located on the outside of the octamere and interact with DNA (fig. 1). Despite their net positive charge histones tend to aggregate, because of hydrophobic regions in the central and carboxy-terminal parts of the molecule. In general, cationic molecules are potent nephritogenic antigens. They can bind to the GBM through charge interactions, penetrate all three layers of the GBM and persist in the GBM provided that their size is between 40 and 500 kD and the pI exceeds 8.5–9.0 [25]. Theoretically, histone aggregates are therefore excellent candidates to act as nephritogenic antigens in vivo, and indeed in 1989 Schmiedeke et al. [26] showed that histones display

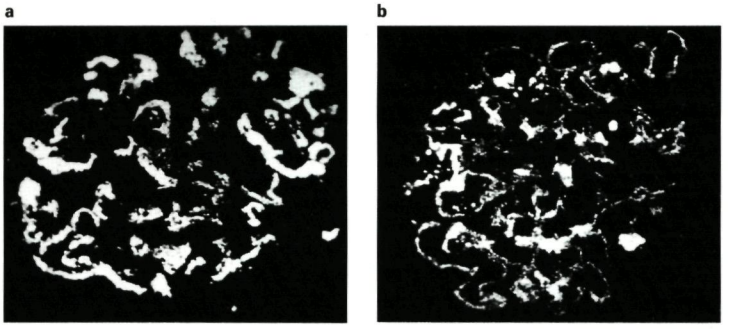

Fig. 2. Direct immunofluorescence of the glomerular binding of an anti-DNA monoclonal antibody after subsequent perfusion of histones, DNA and anti-DNA, showing a granular pattern along the capillary wall (**a**). When the anti-DNA monoclonal antibody is injected intravenously 1 h after renal perfusion of histones and DNA a membranous pattern is observed (**b**). Reproduced, with permission from Termaat et al. [21].

a high affinity for the GBM. To test whether histones and DNA could mediate the binding of anti-DNA in vivo, we performed renal perfusion studies in the rat. In ex vivo perfusion experiments we found that after consecutive perfusion of histones, DNA and purified anti-DNA mAb, the antibodies localized abundantly in a granular pattern along the capillary wall. On immunoelectron microscopy the deposits were localized on the endothelial cells and subendothelially in the GBM. After perfusion of DNA alone and subsequently anti-DNA, we only found glomerular localization with a high avidity mAb, which was, however, much less intense and on electron microscopy exclusively located in the mesangium. Control experiments with histones and anti-DNA or histones, DNA and a nonrelevant mAb (same isotype and concentration), or anti-DNA alone failed to give any glomerular localization. If we changed the perfusion procedure to an in vivo perfusion, in which we administered histones and DNA via the renal artery, restored the renal circulation and then injected 1 h later the purified anti-DNA mAb intravenously, we found a pure membranous localization, with immunoelectron microscopy deposits underneath the visceral epithelial cells and in the slit pores of the GBM [21]. So, anti-DNA antibodies can bind via histones and DNA to the GBM and depending on the perfusion protocol can form subendothelial or subepithelial deposits (fig. 2). Furthermore, only high avidity anti-DNA antibodies can also bind via DNA alone to the mesangium but hardly to the GBM. It is indeed not very likely that DNA, which is negatively charged, binds to the GBM. Izui et al. [27], who developed the idea that DNA can serve as a planted antigen in LN, could only demonstrate binding to the GBM under in vitro conditions. In vivo binding could only be achieved in animals pretreated with LPS [27]. Very recently, it was demonstrated that the injection of LPS leads to the release of nucleosomes in the circulation [28] and this may have mediated the binding of DNA to the GBM in the classical Izui experiments [27]. However, at present it is not known whether nucleosomes themselves can act as planted antigens, since they do not have a net positive charge, although this is not a prerequisite for binding to the GBM. Hybrid molecules without a net positive charge, but with confined cationic regions can bind to the GBM, even despite the presence of anionic charges at other sites of the molecule [29]. This is substantiated by the recent finding that nucleosomes can bind to the glomerulus in vivo [30]. Whether the nucleosome itself, or a partly degraded or altered nucleosome is the nephritogenic particle remains to be elucidated.

Involvement of Histones/Nucleosomes in Lupus Nephritis

Histones have never been described as circulating free molecules, but circulating nucleosomes have been demonstrated in animals treated with LPS or during infections [28], and also in the circulation of SLE patients [31]. Therefore, nucleosomes bound to antibodies may be present in the circulation of SLE patients. As already mentioned we analyzed anti-HS reactivity in SLE patients in relation to clinical disease manifestations. From the data presented so far it is clear that with the HS ELISA anti-DNA antibodies are detected which are complexed to histones and DNA. When we studied anti-HS reactivity in selected SLE patients either with biopsy-proven diffuse proliferative LN or patients with nonrenal exacerbations, in all 5 cases of LN anti-HS reactivity was present before the onset of nephritis, while only 1 of 4 nonrenal exacerbations was positive. In patients who remained clinically stable no anti-HS reactivity was detectable [16]. Subsequently, we analyzed anti-HS reactivity in 72 consecutive SLE patients who experienced 40 exacerbations

over a period of 3 years. In 20 of these exacerbations one or more renal symptoms were present. The patients with evidence of renal involvement had significantly higher anti-HS titers than those without renal manifestations during the flare [17]. When we analyzed the correlation between anti-HS and anti-DNA titers in both studies we found three patterns: anti-DNA-negative sera were always anti-HS negative; certain sera were anti-HS negative despite a high anti-DNA titer, and in the remaining sera there was a significant correlation between the anti-HS and the anti-DNA titer. These findings implicate that anti-HS reactivity is a feature of anti-DNA antibodies and that there may be two subgroups of anti-DNA antibodies: those which do (HS positive) and those which do not (HS negative) complex with nucleosomes. Besides anti-DNA also antihistone antibodies can bind to nucleosomes. Although this review will not analyze in depth the clinical significance of antihistone antibodies in SLE, it became clear, once it was possible to obtain purified histones, that the majority of SLE patients have antihistone antibodies. In some studies a correlation between antihistone reactivity and LN or active disease was described [32, 33], but in other studies this correlation was not found [34–36] and even a negative correlation between the occurrence of antihistone antibodies and LN has been described [37, 38]. Moreover, in drug-induced lupus antihistone antibodies predominate, whereas renal involvement is rare [39]. A possible explanation could be that antihistone antibodies bind to the histone part of the nucleosome in such a way that the interaction between the nucleosome and the GBM is prevented. In contrast to this, anti-DNA antibodies might cover the negative charge of DNA in the nucleosome, thereby enhancing the binding capacity of the nucleosome to the negatively charged GBM.

Histones have been demonstrated in glomerular deposits in human and murine SLE (NZB/WF_1, graft vs. host mice). These deposits could only be identified with the use of antisera against N-terminal regions of histones and not with antisera against other regions of histones, nor with antisera against whole histones [40, 41]. This indicates that the N-terminal regions are the only parts of histones which are accessible in these depositions. This suggests that the nucleosome or the core octamere is grossly intact in these depositions, for the N-terminal regions are located on the accessible outside of the nucleosome. Interestingly, antihistone antibodies in SLE are directed almost exclusively against the same epitopes on the N-terminal part of histones [42]. This suggests that the nephritogenic particle and the immunogen at work in SLE might be the same or resemble each other closely. In both instances it might be the (altered?) nucleosome.

Conclusion

In this review we discussed some recent findings which suggest that histones or nucleosomes play a pivotal role in the initiation of the glomerular inflammation in SLE. In the proposed concept anti-DNA antibodies are complexed to nucleosomes either in the circulation or in situ in the GBM. These nucleosomes are, via their histone part targetted to the GBM through charge interactions with anionic sites. Once these initial deposits are formed, their presence might enhance further deposition of anti-DNA antibodies, via binding to the DNA part of the nucleosome. Recently, indications have emerged (which are not addressed in this review) that nucleosomes might also be involved in the induction of antinuclear antibodies in SLE [43, 44]. It seems therefore that nucleosomes are important for the induction as well as for the pathogenicity of autoantibodies in SLE.

Acknowledgements

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CHAPTER 3

Higher anti-heparan sulphate reactivity during SLE exacerbations with renal manifestations. A long term prospective analysis.

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Higher anti-heparan sulphate reactivity during systemic lupus erythematosus (SLE) disease exacerbations with renal manifestations; a long term prospective analysis

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SUMMARY

Cross-reactive antibodies against heparan sulphate (HS) have been suggested to play a role in initiating renal disease in SLE Recently we found that HS-reactivity is mediated by anti-DNA antibodies complexed with DNA and histones To evaluate the clinical significance of anti-HS reactivity we studied prospectively a cohort of 72 consecutive SLE patients of whom 22 experienced 40 exacerbations In 20 of these exacerbations renal symptoms were present In these 20 exacerbations significantly higher anti-DNA (median 1 160) and anti-HS (median 1 30) titres were detected compared with exacerbations without renal manifestations (median 1 60 for anti-DNA and negative for anti-HS) There were no correlations with other symptoms of SLE Anti-HS titres showed a significant correlation with anti-DNA antibody titres ($r_s = 0\ 57$, $P < 0\ 05$) Anti-HS without anti-DNA reactivity was never detected Some SLE patients showed a high anti-DNA titre without anti-HS reactivity, suggesting that not all anti-DNA antibodies are able to bind to histone/DNA complexes and thus to exhibit anti-HS reactivity Our findings indicate that anti-HS reactivity is correlated with renal disease in SLE

Keywords prospective study systemic lupus erythematosus nephritis anti-DNA antibodies anti-HS reactivity cross-reactivity

INTRODUCTION

SLE is an autoimmune disease characterized by the occurrence of a multitude of autoantibodies primarily directed against nuclear antigens [1] Antibodies against double-stranded DNA (dsDNA) are regarded as a serologic marker for this disease They can be detected in about 70% of SLE patients and in patients with active disease their cumulative incidence is as high as 96% [2] Nephritis is considered to be the most severe manifestation of the disease occurring in about 40–50% of SLE patients The pathogenesis of SLE-nephritis is still controversial Facts are that renal manifestations of SLE are often preceded by a rise in levels of high avidity anti-dsDNA antibodies [2–5] and that these antibodies are detected in considerable amounts in immune deposits in the glomerulus [6] These observations led to the hypothesis that immune complexes containing high avidity anti-dsDNA antibodies and DNA are trapped in the glomerular basement membrane (GBM) thus triggering the glomerular inflammation [7] This hypothesis however, has been the subject of much debate First the existence of circulating DNA/anti-DNA complexes is highly questionable [8] Second, preformed DNA/anti-DNA complexes injected intravenously in experimental animals do not locate in the kidney but instead are rapidly cleared by the liver [9 10] Because of this alternative explanations have been suggested for the initiation of SLE-nephritis [11] which were mainly based on the fact that anti-DNA antibodies can cross-react with several molecules other than DNA [12–14] It was suggested by us [15] and others [11 16] that anti-DNA antibodies can bind directly to intrinsic glomerular antigens This hypothesis was based on the finding that certain anti-DNA antibodies can bind directly to heparan sulphate (HS) an intrinsic constituent of the GBM [15] HS is negatively charged and responsible for the charge-selective permeability of the GBM Later on we found that not anti-DNA antibodies themselves but anti-DNA complexed to DNA and histones are responsible for this cross-reactivity with HS Anti-DNA MoAbs which had the property of binding to HS totally lost this capability after purification on a protein A-Sepharose column under dissociating conditions After reconstitution with the column effluent the MoAbs regained their binding to HS In the reconstituting effluent DNA and histones could be demonstrated [17] Also in SLE sera we demonstrated a strong decrease in anti-HS reactivity after purification under dissociating condi-

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tions [18] We assumed that the histones in the complex were responsible for the binding to HS since histones which are highly positively charged molecules have a high affinity for the GBM [19] In a recent study we showed that if we perfused histones DNA and anti-DNA into a rat kidney we could demonstrate localization of anti-DNA in the GBM while perfusion of anti DNA alone did not lead to binding [20]

The role of histones in SLE-nephritis is further substantiated by the identification of histones in glomerular deposits in human and murine SLE [21 22]

In a retrospective study in a small group of selected SLE patients we found that anti-HS reactivity was correlated with renal disease [18] In order to substantiate this finding in a non-selected consecutive cohort of patients we performed a prospective study in 72 consecutive SLE patients, in whom plasma anti-HS reactivity was correlated with clinical manifestations of disease flares

PATIENTS AND METHODS

Patients and blood sampling

Seventy-two consecutive SLE patients were studied (61 women and 11 men) Details about these patients have been published before [5] They all fulfilled at least four criteria of the American Rheumatism Association (ARA) revised criteria for SLE [1] The mean observation period during this study was 18 5 patient months (range 6–39 months)

A blood sample was obtained at least once a month and more often during periods of active disease These samples were mostly drawn in tubes containing EDTA although in some instances serum was obtained All samples were stored at −20 C

Assessment of disease activity

During disease flares a disease activity index was calculated as described previously [5] This index has been validated by comparing its score on a 0–10 point visual analogue scale, as assessed by clinicians experienced in the management of lupus ($r = 0\,67$, $P < 0\,001$) [5] Criteria for major and minor exacerbations were described previously [5]

Study design

All samples of patients who experienced an exacerbation were analysed in the anti-DNA ELISA and anti-HS ELISA Analysis was performed without knowledge of clinical data In addition to this all samples ($n = 50$) of five patients who remained clinically quiescent during the whole follow-up period were also analysed

Anti-DNA and anti-HS ELISA

The anti-DNA antibody titre was assessed in an anti-DNA ELISA as described previously [18] Briefly, polystyrene microtitre plates (Costar, Cambridge, MA) were precoated with 150 μl/well of protamine chloride (0 5 mg/ml) After washing of the plates, DNA (Calbiochem, San Diego, CA) was coated overnight at room temperature (100 μl/well 50 μg/ml) Plasma samples were diluted in PBS containing 1% w/v gelatin and incubated within the plates for 1 h at 37 C Next, plates were washed and 100 μl of peroxidase-conjugated rabbit anti-human immunoglobulin (ICN Costa Mesa CA) diluted 1 500 in PBS/1 ' gelatin were added per well Plates were incubated for 1 h at 37 C and again washed A freshly prepared substrate solution of 0 8 mg ml 5 aminosalicylic acid dissolved in 50 mM phosphate buffer pH 6 0 supplemented with 0 8 μl ml 30 % v v H_2O_2 was added to each well After 30 min the OD at 450 nm was measured

The anti-HS ELISA was performed in the same way as the anti-DNA ELISA Heparan sulphate (HS Seikaguku Kogyo Ltd Tokyo Japan) 50 μg ml was added to wells precoated with protamine chloride

The titre in the anti-DNA and anti-HS ELISAs was defined as the reciprocal of the dilution of the sample giving an absorption of 1 0 at 450 nm Plasma samples of 50 normal blood bank donors were tested in both ELISAs Cut-off values were defined as the mean $+3 \times$ s d in this group Based on this criterion a sample was considered positive if the titre was > 20 in the anti-HS ELISA and > 10 in the anti-DNA ELISA

Statistical analysis

For statistical analysis Wilcoxon s rank correlation test and Spearman s correlation test were used

RESULTS

Prevalence of disease manifestations during exacerbations

Of the 72 SLE patients who were studied, 22 experienced 40 exacerbations of which 23 were minor and 17 were major Prevalences of disease manifestations during these flares are shown in Table 1 In 20 exacerbations, renal symptoms were present Prevalence of renal symptoms during these exacerbations is shown in Table 2

Anti-HS reactivity in serum versus *plasma samples*

In total, 351 blood samples were obtained, of which 302 were plasma samples and 49 were serum samples Since it is known

Table 1 Incidence of SLE disease manifestations in 40 exacerbations

Manifestation	Number of exacerbations	%
Renal disease	20	50
Vasculitis	11	28
Haematologic disease	22	55
Rash	12	30
Arthritis	20	50
Cerebral	3	8
Serositis	14	35

Table 2 Prevalence of renal symptoms during 20 exacerbations with renal manifestations

Renal symptom	*n*
Proteinuria	14
Erythrocyturia	13
Erythrocyte and/or granular casts	7
Decrease of creatinine clearance	10

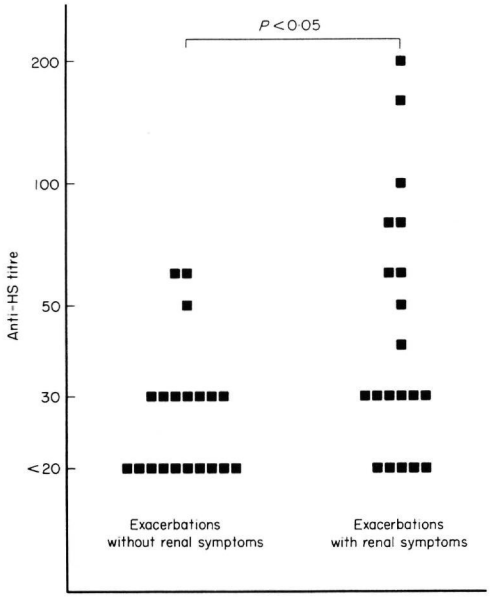

Fig. 1. Anti-heparan sulphate (HS) titres in exacerbations with and without renal symptoms of SLE.

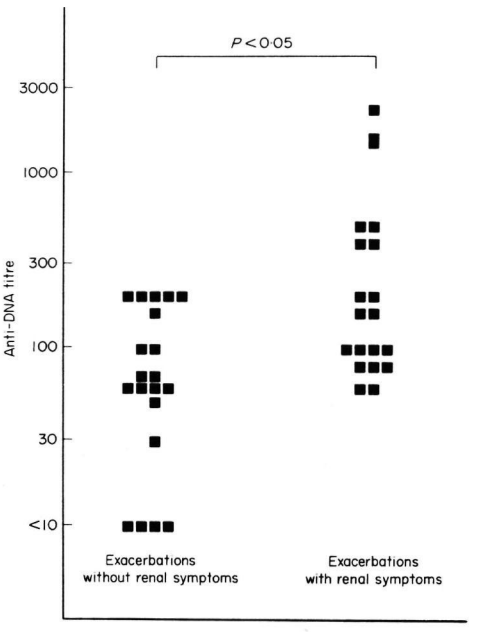

Fig. 2. Anti-DNA titres in exacerbations with and without renal symptoms of SLE.

that DNA and other nuclear antigens can be released during clotting [23], these antigens might bind with anti-DNA antibodies and thus form complexes *in vitro* which falsely might give rise to anti-HS reactivity as an artefact. However, this seems not to be a major problem, since we found previously identical anti-HS reactivity in simultaneously obtained plasma and serum samples [18].

Anti-HS and anti-DNA titres in relation to disease manifestations during exacerbations

When we analysed the levels of anti-HS reactivity during exacerbations, we found higher anti-HS reactivity ($P<0{\cdot}05$) in exacerbations with renal symptoms compared with exacerbations without renal symptoms, although not all exacerbations with renal symptoms were anti-HS-positive (Fig. 1). In contrast, this difference in levels of anti-HS reactivity was not observed when flares were analysed for the presence or absence of other non-renal disease manifestations.

A similar picture emerged when anti-DNA reactivity was compared during disease flares. During exacerbations with renal symptoms, higher anti-DNA reactivity was measured, compared with exacerbations without renal symptoms (Fig. 2). For other, non-renal, symptoms during flares no difference in anti-DNA titre was observed. In the 50 samples obtained from five patients who remained clinically stable during the observation period, we found in only one sample a weak anti-HS reactivity. There was no correlation between severity of disease manifestations and anti-HS or anti-DNA titres, since these titres were not different between minor and major exacerbations. Because the titres were not significantly different between patients with mild ($r\leqslant 3$) or severe ($r\geqslant 4$) renal symptoms, there was also no correlation with the severity of the renal symptoms.

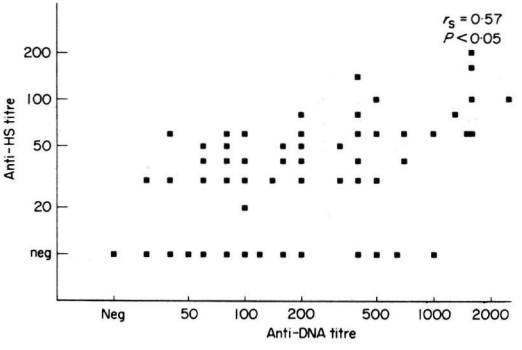

Fig. 3. Anti-DNA titre compared with anti-heparan sulphate (HS) titre in all samples tested. (One point often represents several different measurements.)

Correlation between anti-HS and anti-DNA reactivity

In Fig. 3 the anti-HS reactivity of all samples is plotted against the anti-DNA titre. There is a significant correlation between both titres ($r=0{\cdot}57$, $P<0{\cdot}05$).

DISCUSSION

Based on previous studies in which we found that the cross-reactivity of anti-DNA antibodies with HS [15] was caused by

histone DNA complexes bound to anti-DNA [17] we postulated that this binding could be important for the pathogenesis of SLE-nephritis [24] To elucidate the clinical significance of anti-HS reactivity for SLE-nephritis we have previously evaluated this reactivity longitudinally in a group of 10 patients selected from a cohort of 110 patients on the basis of their clinical history In these patients with first onset of severe diffuse proliferative glomerulonephritis we found anti-HS reactivity in all five renal exacerbations of SLE while only in one out of four non-renal exacerbations was anti HS detected Patients who clinically remained quiescent were negative [18] To discover whether this was also true for a group of non selected SLE patients we analysed anti-HS reactivity in a consecutive cohort of 72 SLE patients who experienced both minor and major disease exacerbations In these patients it was previously found that a rise in anti-dsDNA antibody levels could predict disease exacerbations [5] In the current prospective study we found higher levels of anti-HS reactivity during SLE exacerbations with renal symptoms compared with those without renal symptoms There was no correlation between anti-HS reactivity and any other disease symptom during the flares These findings are in line with a recent study in which antibodies to heparan sulphate proteoglycan were frequently found in high titres in SLE patients with renal involvement [25] In contrast to our previous study in which we found that every exacerbation of SLE-nephritis was preceded by a high anti-HS reactivity we now observed cases with renal symptoms without detectable anti HS reactivity although considerable anti-DNA binding was found We do not have a clear explanation for this discrepancy It may be related to differences in selection of patients In the first study only patients with severe and first onset of SLE-nephritis were analysed In the present study SLE patients were analysed who had already had their disease for a variable time period and had experienced previous flares with renal symptoms This suggests that there might be a difference in the pathogenic significance of HS-reactive antibodies for the initial glomerular damage and subsequent episodes of glomerular inflammation HS-reactive anti-DNA antibodies are due to antibodies complexed in the circulation to histone/DNA particles This is in line with the recent finding that histones and DNA are present in the circulation as oligonucleosomal particles [26 27] These HS-reactive circulating complexes are targeted to the GBM via their histone part inducing glomerular deposits The anti-DNA antibodies that do not react with HS may localize in the glomerulus via an *in situ* immune complex mechanism once histone/DNA particles have bound previously to the GBM This view is supported by the recent finding that some anti-DNA antibodies form circulating immune complexes which can localize in the GBM while others deposit via an *in situ* immune complex mechanism [28] These different binding mechanisms might explain why in some instances renal symptoms may ensue in the absence of HS-reactive complexes in the circulation but in the presence of non-HS-reactive anti-DNA antibodies

As previously [18] we also found in this study that anti-HS reactivity is not a mere reflection of the anti-DNA titre We never found anti-HS reactivity in the absence of anti-DNA reactivity (Fig 3) which stresses again that anti-HS reactivity is mediated by anti-DNA antibodies and that there are no autoantibodies in SLE which specifically and directly bind to HS as suggested by others [29 30] From Fig 3 it also becomes clear that there is a subgroup of anti DNA antibodies which despite a high anti DNA titre does not bind to HS In contrast to this another group of anti DNA antibodies shows HS reactivity and the titres of these reactivities are correlated Hypothetically these two subgroups of antibodies can contribute in a different way to glomerular damage as discussed before

In this prospective study we found a correlation between renal symptoms during SLE disease flares and anti-HS reactivity Autoantibodies complexed to histones and DNA displaying HS reactivity might contribute to the glomerular inflammation in lupus nephritis Future research has to reveal in more detail the inflammatory potential of these antibodies in the glomerulus

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CHAPTER 4

Anti-nucleosome antibodies complexed to nucleosomal antigens show anti-DNA reactivity and bind to rat glomerular basement membrane in vivo.

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Anti-nucleosome Antibodies Complexed to Nucleosomal Antigens Show Anti-DNA Reactivity and Bind to Rat Glomerular Basement Membrane In Vivo

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Abstract

Histones can mediate the binding of DNA and anti-DNA to the glomerular basement membrane (GBM). In ELISA histone/DNA/anti-DNA complexes are able to bind to heparan sulfate (HS), an intrinsic constituent of the GBM. We questioned whether histone containing immune complexes are able to bind to the GBM, and if so, whether the ligand in the GBM is HS. Monoclonal antibodies (mAbs) complexed to nucleosomal antigens and noncomplexed mAbs were isolated from culture supernatants of four IgG anti-nuclear mAbs. All noncomplexed mAbs showed strong anti-nucleosome reactivity in ELISA. One of them showed in addition anti-DNA reactivity in noncomplexed form. The other three mAbs only showed anti-DNA reactivity when they were complexed to nucleosomal antigens.

After renal perfusion a fine granular binding of complexed mAbs to the glomerular capillary wall and activation of complement was observed in immunofluorescence, whereas noncomplexed mAbs did not bind. Immuno-electron microscopy showed binding of complexes to the whole width of the GBM. When HS in the GBM was removed by renal heparinase perfusion the binding of complexed mAb decreased, but did not disappear completely.

We conclude that anti-nucleosome mAbs, which do not bind DNA, become DNA reactive once complexed to nucleosomal antigens. These complexed mAbs can bind to the GBM. The binding ligand in the GBM is partly, but not solely, HS. Binding to the GBM of immune complexes containing nucleosomal material might be an important event in the pathogenesis of lupus nephritis. (*J. Clin. Invest.* 1994. 94:568–577.) Key words: systemic lupus erythematosus • nephritis • anti-nucleosome • histones • heparan sulfate

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Introduction

Systemic lupus erythematosus (SLE)[1] is an autoimmune disease characterized by the occurrence of numerous auto-antibodies directed primarily against nuclear antigens Antibodies against double-stranded DNA (dsDNA) are regarded as highly specific for the disease (1) Nephritis is one of the most serious manifestations of the disease Since nephritis is associated with a rise in titer of especially high avidity IgG anti-dsDNA and a depression of serum complement, it is generally assumed that these antibodies are involved in the pathogenesis of lupus nephritis and that they cause complement activation (2–4) Moreover, enrichment of these antibodies in renal and glomerular eluates of SLE patients and lupus mice has been found, sustaining the nephritogenic properties of these antibodies (5–7) How these antibodies bind in the glomerulus, however, is still unclear The classical hypothesis that anti-DNA is deposited in the glomerular basement membrane (GBM) as DNA/anti-DNA immune complexes has been subject of much controversy (8) For instance, when artificially prepared DNA/anti-DNA immune complexes are injected in experimental animals, they show no affinity for the GBM, but instead are rapidly cleared by the liver (9–11) Indeed, it is not very likely that the negatively charged DNA in anti-DNA/DNA complexes would easily deposit in the negatively charged GBM (12) This controversy led Schmiedeke et al (13) to the idea that binding of histones, which are highly positively charged DNA binding proteins might mediate the binding of DNA and anti-DNA At the same time, analyzing the cross-reactivity of anti-DNA antibodies with heparan sulfate (HS), an intrinsic constituent of the GBM, we found that this cross-reactivity was due to histone/DNA containing immune complexes (14) The histone part in these complexes was responsible for the binding to HS Furthermore, when we perfused subsequent histones, DNA and anti-DNA in a rat kidney, we observed a strong fine granular binding of antibodies to the capillary wall in immunofluorescence (IF), whereas after perfusion of DNA and anti-DNA, the binding was much less and restricted to the mesangium (15) The concept that histones may play a role in the initiation of lupus nephritis (reviewed recently in reference 16) was further substantiated

1 *Abbreviations used in this paper* dsDNA, double-stranded DNA, GBM, glomerular basement membrane, HS, heparan sulfate, GAG, glycosaminoglycan, HSPG, heparan sulfate proteoglycan, IF, immunofluorescence, IEM, immuno-electron microscopy, NGS normal goat serum, PLP, a mixture of 0 01 M sodium periodate 0 075 M lysine HCl 0 0375 M Na_2HPO_4 and 2% paraformaldehyde, SLE, systemic lupus erythematosus

by the finding of histone deposits in human and murine SLE nephritis (17, 18).

We realized however, that subsequent perfusion of purified histones, DNA and anti-DNA is a rather artificial system. It is not likely for histones and DNA to exist as separate entities in vivo. Indeed, in the circulation DNA is present bound to histones forming oligonucleosomal complexes (19, 20). When such nucleosomal material is released in the circulation of SLE patients, it is very likely that immune complexes are formed with anti-nuclear antibodies with specificity for either histones, nucleosomes or DNA. As we described before (14, 21) a similar complex formation occurs in supernatants of hybridomas secreting anti-nuclear antibodies. In these supernatants mAbs can be found complexed with nucleosomal antigens released from dying cells. That is why we chose to isolate immune complexes from such culture supernatants, to find out which characteristics of these immune complexes are associated with binding capacity in the GBM.

Methods

Production of monoclonal antibodies

mAbs were obtained by fusion of spleen cells from mice with spontaneous ([NZB × W]F1, MRL/Mp/Ipr/Ipr) or experimentally induced (parental-F1 chronic Graft Versus Host disease) SLE and screened for the presence of anti-dsDNA reactivity in ELISA or Farr assay as described previously (22) and new culture supernatants were prepared from which antibodies were purified. For the studies described in this paper, mAbs were selected which showed reactivity with HS in crude culture supernatants, since this reactivity is due to mAbs complexed to nucleosomal material (histones and DNA) as described previously (14). All selected mAbs belong to the IgG2a subclass.

Preparation of immune-complexed and pure mAbs

Hybridomas were grown in serum free conditions (IMDM; GIBCO-BRL, Paisley, UK) supplemented with serum-free medium supplement, SF1 (Costar, Cambridge, MA) 2% vol/vol. Three purification procedures were used.

Procedure I (physiological conditions). Culture supernatants were applied to a protein–A Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with phosphate buffered saline (PBS).

Procedure II (high salt conditions). NaCl, glycine and NaOH were added to culture supernatants to a final concentration of 3 M NaCl, 1.5 M glycine, pH 8.9, before applying to a protein–A Sepharose column which was equilibrated with a 3 M NaCl, 1.5 M glycine, pH 8.9 buffer.

Procedure III (DNase and high salt conditions). In this procedure the purification under high salt conditions was preceded by a DNase treatment. The culture supernatants were first treated with DNaseI (Boehringer Mannheim, Mannheim, Germany) with a final concentration of 40 μg/ml in the presence of 10 mM $MgCl_2$. After 1 h incubation at 37°C the reaction was quenched with EDTA (Merck, Darmstadt, Germany) with a final concentration of 15 mM.

After each of these three different purification procedures immunoglobulins bound to the protein A column were eluted with a solution of 0.1 M citric acid, pH 2.6. The eluate was immediately brought to pH 7.4, dialyzed against PBS and stored at −20°C.

Using the third procedure, noncomplexed mAbs were obtained except for mAb No. 34. For this mAb still a fraction of the antibodies was complexed to nucleosomal antigens. The complexed and noncomplexed antibodies were separated with the use of a DNA-cellulose column (P-L Biochemicals, Inc., Milwaukee, WI), which was equilibrated with PBS. Complexed antibodies retained on the column, whereas pure, noncomplexed antibodies were obtained in the column effluent.

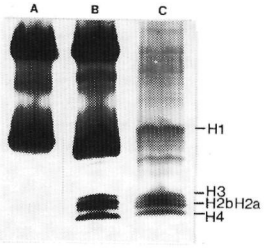

Figure 1. SDS-PAGE of pure and complexed mAbs. (Lane *A*) Pure mAb No. 32 obtained via procedure III (only the heavy and light chains of Ig, but no histone bands are present). (Lane *B*) Complexed mAb No. 32 obtained via procedure I, containing both the Ig heavy and light chains and the four core histones, but not H1. (Lane *C*) Mixture of histones (Boehringer Mannheim). The H1, H2a, H2b, H3, and H4 bands are all present, and there is some contamination with other proteins in this histone mixture.

Characterization of bound antigens in immune-complexed mAbs

The presence of histones in complexed mAbs was determined by SDS-PAGE (8–25%) analysis followed by double silver staining (Phast gel system, Pharmacia). Histones were further identified by immunoblotting. The proteins were transferred to nitrocellulose by means of diffusion at 50°C using the Phast gel system (Pharmacia). Histones were identified using a rabbit anti-histone polyclonal antiserum diluted 1:100, prepared in our laboratory (15). The blot was developed with peroxidase-conjugated goat anti–rabbit Ig (ICN, Costa Mesa, CA) and stained with 4-chloro-1-naphtol. For the identification of DNA in the complexed mAbs, DNA was extracted by phenol-chloroform after proteolysis with proteinase K (Boehringer Mannheim). Proteolysis was performed by adding 100 μg proteinase K in 5 μl water to 300 μg Ig, for 1 h at 50°C. The DNA extract was 5′ end-labeled with [^{32}P]dATP. Labeling was performed according to standard protocols using T4 polynucleotide kinase. After labeling DNA was analyzed on a 6% polyacrylamide gel (Sequagel-6; National Diagnostics, Atlanta, GA) using a labeled 123 bp ladder (Pharmacia) as a size marker.

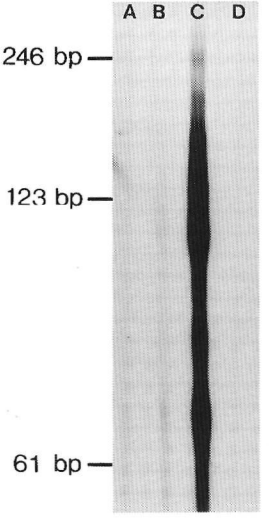

Figure 2. PAGE of DNA extract after labelling with [^{32}P]dATP. Three purification procedures were used on culture supernatants of mAb No. 32. In lane *A* hardly any DNA in the pure mAb (procedure III) is present. In complexed mAb No. 32, (procedure II) small amounts of DNA are detectable (lane *B*). Only after purification according to procedure I (lane *C*) large quantities of DNA are present in the complexed mAb. In lane *D* a purified control mAb (WT1 mouse anti–human CD7) is shown in which no DNA is detectable.

Table I Composition, In Vitro Reactivity, and Glomerular Binding of the Different mAb Preparations

	No 2			No 32			No 34			No 42
mAb nr procedure	I	II	III	I	II	III	I	II	III	II
Composition										
Histones*	+	+	−	++	++	−	++	+++	−	−
DNA‡	+	±	−	++	±	−	+	±	−	−
In vitro reactivity										
Farr§	6	25	3	70	75	3	17	57	3	92
ELISA¶										
DNA	178	227	36	98	100	1	178	200	16	25600
Histone	55	90	< 1	4500	187	1	1777	800	494	< 1
Nucleosome	3500	4800	3000	5000	7600	5400	7500	12000	12000	10000
HS	11	36	4	9	9	1	13	20	1	< 1
Glomerular binding‖	+	+	−	++	+++	−	++	+++	−	−

Composition of bound complexes (*Composition*), reactivity in Farr assay and various ELISAs (*In vitro reactivity*) and glomerular binding (*Glomular binding*) of the different mAb preparations obtained after purification under physiological conditions (I) high salt conditions (II), or DNase treatment followed by high salt conditions (and in the case of mAb No 34 additional separation of histone bound mAb on a DNA-cellulose column, see text) (III) * Histone content as assessed on SDS-PAGE, the intensity of the histone staining was scored semiquantitatively by comparing the intensity of the staining with that of the heavy and light chain ‡ Presence of DNA fragments (of predominately 120 bp) as assessed after labeling with ^{32}P § Percent precipitation of labeled DNA per 12 μg of IgG, values ≤ 3% are regarded as negative ¶ Titer/mg IgG ‖ Glomerular binding was assessed with indirect IF

The immunoglobulin (Ig) concentration was determined in an ELISA, for which Nunc-immuno plates (Maxisorp F96, GIBCO BRL) were coated overnight at 4°C with goat anti-mouse Ig (Sigma Chemical Co, St Louis, MO) diluted 1 80 in PBS, 100 μl per well Plates were washed five times with PBS containing 0 05% (vol/vol) Tween 20 and blocked with PBS containing 1% wt/vol gelatin for 2 h at 37°C Samples were serially diluted in PBS/1% gelatin and incubated for 1 h at 37°C Next, plates were washed again and 100 μl of peroxidase-conjugated goat anti-mouse IgG2a diluted 1 750 (Southern Biotechnology Assoc Inc, Birmingham, AL) in PBS/1% gelatin were added per well After 1 h of incubation at 37°C, the plates were washed A freshly prepared substrate solution of 0 8 mg/ml 5' aminosalicylic acid dissolved in 50 mM phosphate buffer, pH 6 0, supplemented with 0 8 μl/ml 30% vol/vol H_2O_2 was added to each well After 30 min the OD at 450 nm was measured An IgG2a myeloma protein (RPC 5, Cappel, Organon Technika NV, Turnhout, Belgium) was used as standard

Tests for antigen reactivity of purified and complexed anti-nuclear mAbs

Anti-dsDNA assays Anti-dsDNA activity was determined in the Farr assay and in an anti-dsDNA ELISA The Farr assay was performed as described previously (23) In the anti-dsDNA ELISA photobiotinylated PUC9 plasmid DNA was used (24, 25) Briefly, plates (Maxisorb F96, GIBCO-BRL) used for the ELISA were coated overnight with streptavidin (Sigma Chemical Co) diluted in distilled water (150 μg in 150 μl/well) at 4°C The plates were washed three times in PBS, containing 0 05% Tween 20 and three times with distilled water Next, the plates were coated overnight with photobiotinylated DNA (1 μg/ml in PBS, 4°C, 150 μl/well) The plates were washed again and blocked for 2 h at room temperature with PBS containing 10% (vol/vol) normal goat serum (NGS) The use of this blocking agent did not influence the binding of the different mAb preparations when compared to gelatin or BSA, excluding interference by putatively present nucleosomes or nucleohistone This was further substantiated by the fact that mAbs with a specificity restricted to nucleosomes did not bind to plates coated with the batch of NGS used for blocking Samples were serially diluted in PBS, 0 05% Tween 20, containing 10% NGS and incubated for 2 h at room temperature Next, the plates were washed again and 100 μl of peroxidase labeled rat mAb anti-mouse Ig (CLB-RM-19, CLB, Amsterdam, the Netherlands) diluted 1 1,000 in PBS were added per well The plates were washed again and developed with 3,5,3',5'-tetramethylbenzidine (Merck), 100 μg/ml in 0 11 M sodium acetate, pH 5 5, containing 0 003% H_2O_2 By adding 2 M H_2SO_4, 100 μl/well, color development was stopped after 15 min and OD at 450 nm was measured

Anti-HS ELISA HS, purified from bovine kidney, was purchased from Seikagugu Kogyo Ltd (Tokyo, Japan) Photobiotinylation of HS was performed in the same way as described for DNA (25) The concentration of HS was determined by the Farndale assay (26) The ELISA was performed identically as described for DNA A mAb against HS (JM 403, described in reference 27) was used as a positive control

Anti-histone ELISA A mixture of the five histones (H1, H2a, H2b, H3, and H4, 2 5 μg/ml Boehringer Mannheim) in 0 1 M Glycine/NaOH, pH 9, 0 1 M NaCl was coated overnight at 4°C to Nunc-immuno plates The plates were washed five times with PBS/0 05% Tween 20 and blocked with 5% (vol/vol) fetal calf serum (FCS) in PBS 150 μl/well, for 1 h at room temperature Using the same methods as described for NGS we could exclude that the use of FCS influenced the results The plates were washed again and samples serially diluted in PBS/0 05% Tween 20, containing 5% FCS, were incubated (100 μl/well) for 2 h at 37°C After washing the plates again, the procedure was identical to that for the anti-DNA ELISA

Anti nucleosome ELISA Nucleosomes were a kind gift of Dr L Cebecauer (Research Institute of Rheumatic Diseases, Piešťany, Slovakia) They were purified from calf thymus according to standard protocols (28) and contained all five histones and no other proteins (as assessed by SDS-PAGE analysis) and DNA (as assessed on an agarose gel, data not shown) Nunc-immuno plates were coated with nucleosomes 2 5 μg/ml, in 0 15 M NaCl, 0 15 M trisodium citrate, pH 7 0, for 1 h at room temperature (100 μl/well) Plates were washed five times with distilled water and samples, serially diluted in PBS/0 05% Tween 20, containing 0 1% highly purified human serum albumin were

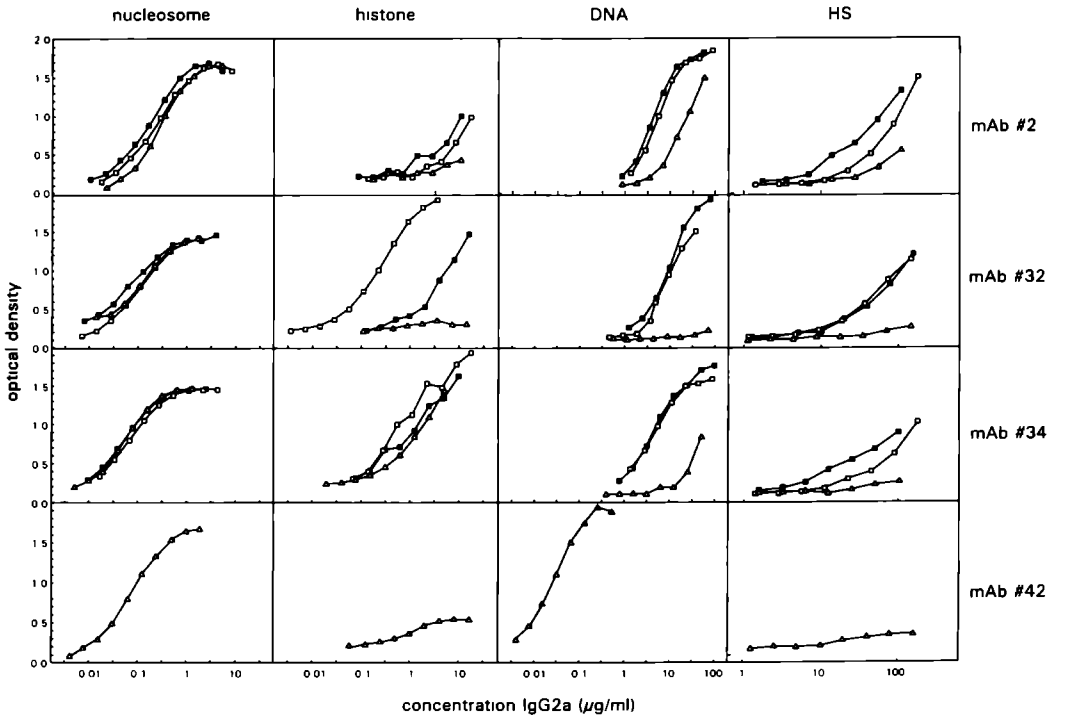

Figure 3 Reactivity of all described antibody preparations in the different ELISAs displayed over a wide range of antibody concentrations The open triangles represent pure noncomplexed antibodies open rectangles represent immune complexes containing both histones and DNA and closed rectangles represent immune complexes containing histones and only a small amount of DNA (see Table I *Composition*) For mAbs No 2, 32, and 34 pure noncomplexes antibodies only react with the intact nucleosome and pure noncomplexed mAb No 42 reacts with both nucleosomes and DNA The other three mAbs show only DNA reactivity once complexed to nucleosomal material

incubated 100 μl/well, for 1 h at room temperature After washing the plates five times with PBS/0 05% Tween, further procedures were identical to the anti-DNA ELISA

All ELISAs were titrated and the reciprocal of the dilution leading to an absorption of 1 0 at 450 nm was used as titer All titers were expressed per mg of IgG2a

Preparation of reconstituted immune complexes

Purified nucleosomes (30 μg in 250 μl PBS/0 1 mM EDTA-1% BSA) were mixed with non-complexed mAb No 32 (250 μg in 250 μl PBS-1% BSA) obtained after purification procedure III This mixture was incubated for 1 h at room temperature and used for renal perfusion studies in rats

Renal perfusion studies

Male Wistar rats (150 g) were anesthetized by intraperitoneal administration of 0 15 ml (9 mg) sodium pentobarbital (Narcovet Apharmo, Arnhem, The Netherlands)

The renal perfusion was performed as described before (15) After perfusion the perfused kidney was taken out and immediately snap frozen in liquid N_2 for immunofluorescence (IF)

In some experiments only a small part of the kidney was taken for IF The remaining part was perfused with PLP for 10 min and prepared for immuno electron microscopy (IEM) (for details, see below)

In additional experiments, after placing a ligature around the aorta between the origins of the left and the right renal artery, a 24 gauge catheter was placed in the aorta and the vena cava was not incised The left kidney was flushed with 0 3 ml 1% (wt/vol) BSA in PBS, followed by perfusion of pure mAb or immune complexes (150–500 μg in 500 μl), which were allowed to bind for 3 min After flushing the left kidney again with 0 3 ml PBS 1% BSA, the ligature and catheter were removed to re-establish normal blood flow

After 15 min the ligature was placed again and an 18 gauge catheter was placed in the aorta and the left kidney was flushed for 3 min with PBS-1% BSA, before it was taken out and snap frozen in liquid N_2 or prepared for IEM by PLP perfusion

Heparinase perfusion

After placing the ligature and insertion of an 18 gauge catheter, the left kidney was flushed with saline for 2 min Next heparinase III (Sigma Chemical Co) 60 U in saline containing 1% BSA and 5 mM $CaCl_2$ was perfused at a flow rate of 1 ml/min The enzyme was allowed to act for 4 min Next the kidney was flushed with PBS-1% BSA for 3 min This heparinase perfusion was followed by perfusion of complexed mAbs The kidney was further processed as described above To control for nonspecific changes perfusion was performed with buffer without heparinase and subsequent with complexed mAbs Experiments were repeated three times with different amounts of perfused complexed

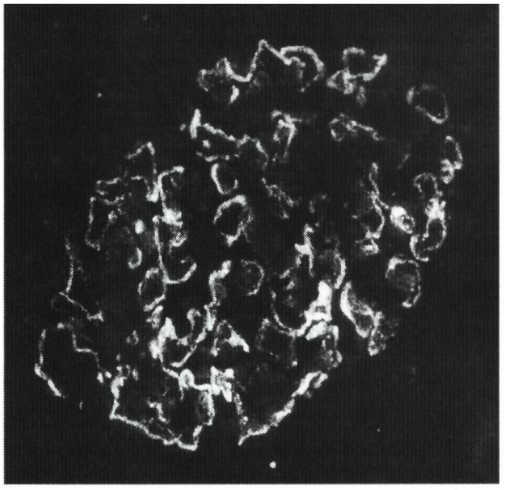

Figure 4. Direct immunofluorescence of the glomerular binding after perfusion with mAb No. 32 complexed to histones. Binding of complexed mAb in the capillary wall of the glomerulus can be observed (×600).

mAbs (ranging from 200–600 μg IgG2a). The expression of glomerular HS was assessed with IF by the anti-HS mAb JM 403 (described in reference 27).

Immunohistology

Immunofluorescence. Direct IF was performed on 2-μm cryostat sections from perfused rat kidneys using a F(ab)$_2$ FITC-labeled sheep anti–mouse IgG (Cappel, Organon Technika NV) diluted 1:750 in order to detect deposition of perfused mouse Ig. Sections were also stained with a rabbit anti histone polyclonal antiserum diluted 1:600. This antiserum was prepared as described before (15). After rinsing with PBS bound rabbit antibodies were visualized using a FITC-labeled mouse anti–rabbit IgG(H + L) diluted 1:40 (Jackson Laboratories Inc., West Groove, PA). In experiments in which the blood flow was restored after perfusion, sections were also stained with FITC-labeled goat anti–rat C3 diluted 1:40 (Nordic, Tilburg, the Netherlands).

In the experiments in which heparinase was perfused before perfusion of complexed mAbs, sections were stained with a mouse IgM anti–HS-glycosaminoglycan (HS-GAG) side chain mAb (JM 403, described in reference 27) and with a goat anti–human HS-core protein antiserum cross reacting with rat HS-proteoglycan (HSPG, described in reference 29). As secondary antibodies FITC labeled goat anti–mouse IgM (Fc) (Nordic) diluted 1:100 or FITC-labeled rabbit anti–goat Ig (De Beer Med. BV, Hilvarenbeek, the Netherlands) diluted 1:500 were used. The sections were also stained with a rabbit anti-rat (L2) laminin polyclonal antiserum diluted 1:400 and with a goat anti–human collagen IV polyclonal antiserum cross-reacting with rat diluted 1:200, which were both prepared in our laboratory, followed by FITC-labeled mouse anti–rabbit Ig (Jackson Laboratories Inc.) diluted 1:40 and by FITC labeled rabbit anti–goat Ig (de Beer Med BV) diluted 1:500, respectively, as second antibodies. Deposition of mouse Ig was evaluated using a F(ab)$_2$ FITC-labeled sheep anti–mouse IgG as described. The Ig depositions were semiquantitatively scored on blinded sections in 30 glomeruli per kidney on a scale from 0–4+ by two independent observers.

After all staining procedures, sections were embedded in Aquamount (BDH Ltd, Poole, England) and examined with a Zeiss fluorescence microscope.

Immuno-electron microscopy. Tissue was prepared for IEM by perfusion of the kidneys during 10 min with a mixture of 0.01 M sodium periodate, 0.075 M lysine HCl, 0.0375 M Na_2HPO_4 and 2% paraformaldehyde (PLP). Thereafter the kidney was cut into small pieces (5 × 5 × 1 mm) and immersed in PLP for another 3 h. Next, the tissue was washed in PBS for 30 min and cryoprotected by immersion in 80% wt/vol sucrose in PBS for 1 h, before they were snap frozen in liquid N_2.

20-μm sections were incubated with a peroxidase labeled anti–mouse Ig diluted 1:20 (Dakopatt, Copenhagen, Denmark). After three washes in PBS, the sections were preincubated in diaminobenzidine medium containing 0.6 wt/vol TRIS for 10–15 min, followed by diami-

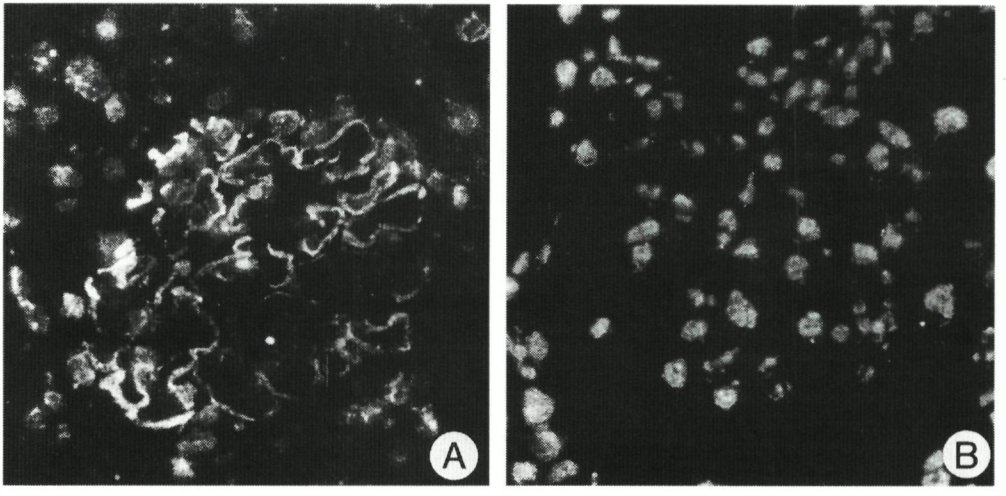

Figure 5. Indirect immunofluorescence of histones in a kidney perfused with mAb No. 32 complexed to histones (*A*). A positive staining is seen in the nuclei and along the glomerular capillary wall. After perfusion with pure mAb No. 32 only nuclear staining is observed (*B*) (×500).

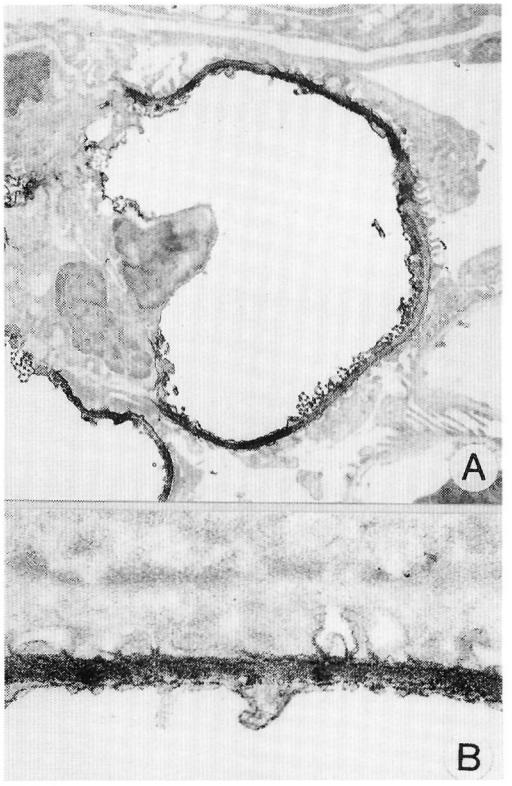

Figure 6. Immuno-electron microscopy after perfusion with mAb No. 32 complexed to histones. There is binding of complexed mAb throughout the width of the GBM and only limited deposition on mesangial or endothelial cells (*A*). In a detail (*B*) the GBM binding of these complexes is visualized more clearly (*A*, ×5,000; *B*, ×20,000).

nobenzidine with addition of H_2O_2 to a final concentration of 0.001% vol/vol. The sections were washed in distilled water, postfixed in 0.1 M phosphate buffered 2% wt/vol OsO_4 for 30 min at room temperature, dehydrated, and embedded in Epon 812. Thin sections were prepared on a LKB ultratome, and examined unstained in an electron microscope (Jeol 1200 EX2, Jeol Europe BV).

Results

Identification of histone and DNA in complexed and purified mAbs. From a panel of mAbs originally identified as anti-DNA mAbs (described in reference 22) mAbs No. 2, 32, 34, and 42 were used in this study. Purified noncomplexed mAbs were obtained after procedure III for mAbs No. 2 and 32 while for mAb No. 34 this procedure had to be supplemented by purification on a DNA–cellulose column. For mAb No. 42 noncomplexed antibodies were obtained after procedure II. Immune complexes containing the core histones (H2a, H2b, H3, and H4) and DNA were obtained after procedure I, except for mAb No. 42 which did not bind protein A under physiological conditions. The presence of histones was visualized by SDS-PAGE (Fig. 1, showing the results for mAb No. 32) and immunoblotting (data not shown). DNA fragments were demonstrated in the complexed mAb after labeling with ^{32}P. The greater part of this DNA had a size of ~ 120 bp but also some larger (240 bp) and smaller fragments were seen (Fig. 2). Immune complexes containing histones and only a small amount of DNA were obtained after procedure II for mAbs No. 2, 32, and 34. A summary of the composition of the complexed material bound to the mAbs is given in Table I (*Composition*).

Reactivity of complexed and pure mAbs in ELISA and Farr assay. The reactivities of the different preparations of these mAbs in the Farr assay and in the different ELISAs are given in Table I (*In vitro reactivity*). In Fig. 3 the results of the different ELISAs are displayed in a dose effect manner over a wide range of antibody concentrations. Anti-DNA reactivity in the Farr assay and in the ELISA was found after purification under physiological conditions (procedure I) and comparable with that observed in crude culture supernatants (data not shown). DNA binding became even greater after reduction of the amount of DNA in the complex (procedure II). After complete removal of bound nuclear material from these mAbs, mAbs No. 2, 32, and 34 lost their anti-DNA reactivity almost completely, whereas No. 42 remained strongly positive. A more or less similar picture emerged when the different preparations were tested on histones, i.e., positive in complexed form and negative in purified form, except for mAb No. 34. Completely different was the reactivity towards nucleosomes, the reactivity remained unaltered after purification. From these data it can be concluded that mAb Nos. 2 and 32 recognize an epitope expressed only on the intact nucleosome, mAb No. 34 reacts with an epitope on the intact nucleosome although in pure form it also reacts with histones, suggesting that for the recognition of the epitope on nucleosomes histone related antigens are involved. Finally, mAb No. 42 primarily reacts with dsDNA, which also explains the reactivity with the intact nucleosome.

Glomerular binding after perfusion of complexed and pure mAbs. The glomerular binding that occurred after renal perfusion of the different mAb preparations is summarized in Table I (Glomerular binding). From these perfusion experiments it becomes clear that binding only occurs if the mAbs are complexed. A representative example of the observed binding is given in Fig. 4. In purified form none of the mAb showed any binding. The major determinant for GBM binding was the presence of histones in the complex, irrespective of the amount of DNA present. In fact for mAbs No. 32 and 34 the magnitude of the binding was even larger for preparations obtained after purification under high salt conditions (procedure II, reducing the amount of bound DNA considerably), compared with preparations obtained after purification under physiological conditions (procedure I; not removing the DNA from the nucleosomal particle). This suggests that reduction of the DNA content within the nucleosome enhances the glomerular binding. The involvement of histones in this GBM binding is further substantiated by indirect IF using an anti-histone antiserum showing besides nuclear staining, a fine granular deposition of histones along the capillary wall after perfusion of complexed mAb (Fig. 5 *A*), but no capillary wall staining after perfusion of pure noncomplexed mAb (Fig. 5 *B*). At the ultrastructural level the Ig binding occurred through the whole width of the GBM, with only very limited binding to the endothelium or the mesangium

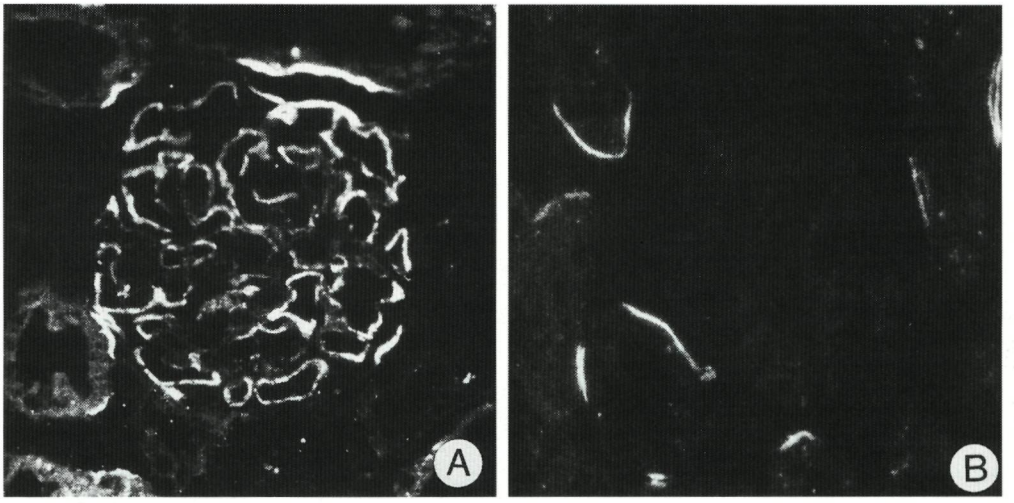

Figure 7. Direct immunofluorescence for rat C3 after perfusion with mAb No. 32 complexed to histones, followed by restoration of the blood flow for 15 min. Note deposition of rat C3 in the capillary wall of the glomerulus (*A*). When pure mAb No. 32 was perfused followed by restoration of the blood flow, there was no deposition of rat complement in the glomerulus (*B*), only staining of the basal part of the proximal tubular cells, which is normal in rat kidneys (×480).

(Fig. 6, *A* and *B*). That this glomerular binding may have pathogenic potential can be derived from the observation that restoration of the renal circulation after perfusion of complexed mAbs leads to C3 deposition along the capillary wall in a pattern identical to that of the Ig deposits (Fig. 7).

Glomerular binding after perfusion of nucleosome reconstituted complexes. When purified mAbs were reconstituted with purified nucleosomes, glomerular binding was observed in IF after perfusion, which was comparable with the binding seen after perfusion of complexed antibodies obtained from culture supernatants. A representative example of the observed binding is given in Fig. 8. When the noncomplexed mAb, used for the reconstitution of these immune complexes, was perfused, no binding was observed, indicating that it was the nucleosome in the complex, which mediated the glomerular binding of the antibodies. In addition perfusion of nucleosomes with a nonrelevant mAb WT1 directed against human CD7 did not lead to glomerular antibody binding.

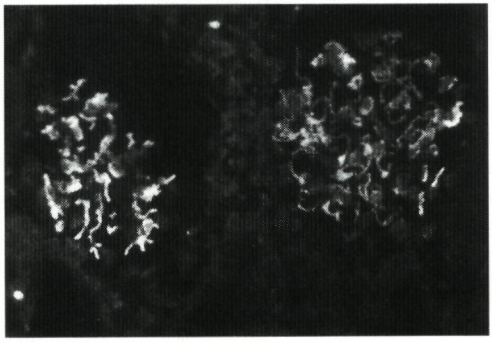

Figure 8. Direct immunofluorescence of the glomerular binding after perfusion of a reconstituted immune complex of pure noncomplexed mAb No. 32 (obtained after procedure III) and highly purified nucleosomes. Binding of complexed mAb in the capillary wall of the glomerulus can be observed (×300).

Binding of complexed mAbs after heparinase perfusion. To investigate the significance of GBM-HS in the glomerular binding of complexed mAb, heparinase was perfused before mAb perfusion. After heparinase, staining of the HS-GAG side chain within the GBM had almost disappeared completely, whereas staining of the HSPG-core protein was intact, indicating that the HS-GAG side chain was effectively removed from the core protein by this procedure (Fig. 9). Staining of other GBM components, like laminin or collagen IV also remained intact (data not shown). If histone-containing immune complexes were perfused after heparinase perfusion, binding of the mAb decreased, but did not disappear totally (Table II, Fig. 9), suggesting that HS is not the only ligand through which these immune complexes can bind to the GBM as we have already suggested before (15).

Discussion

In previously reported experiments from others (13) and from our laboratories (14, 15, 30), it was shown that anti-dsDNA antibodies can bind to the GBM via an intermediate bridge of histones and DNA, in which the histone part is responsible for the binding to anionic sites in the GBM (for review see reference 16). Since our previous studies were performed with subsequent separate perfusion of histones, DNA, and purified anti-DNA, we now extend these observations in the present study by using mAb to nucleosomal antigens. These mAbs were se-

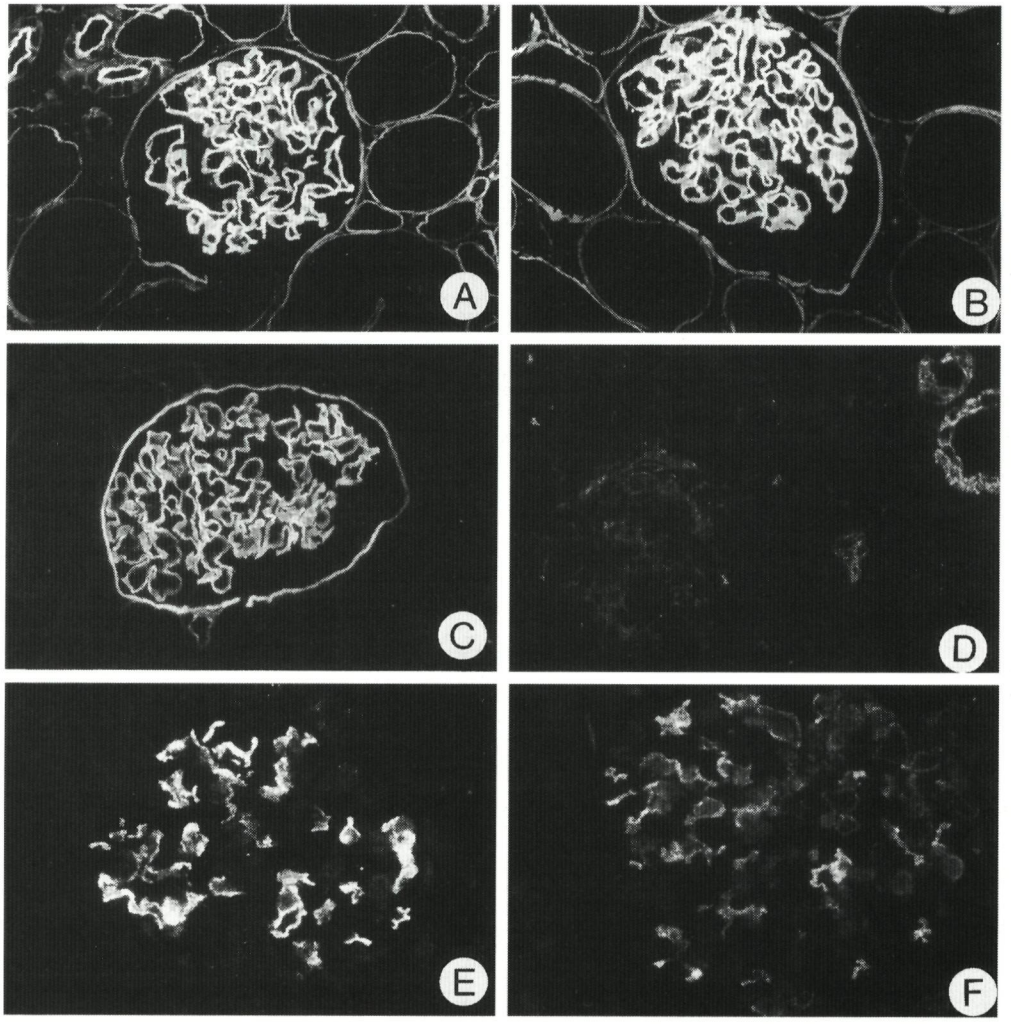

Figure 9. Indirect immunofluorescence of a kidney perfused with heparinase compared with a control perfusion. After control perfusion the staining of HSPG-core protein (*A*) and HS-GAG side chain (*C*) is intact. In the kidney perfused with heparinase the staining of the HSPG-core protein is still intact (*B*), whereas the staining of the HS-GAG side chain has disappeared (*D*). When mAb No. 32 complexed to histones is perfused after heparinase or a control perfusion, the binding of complexed mAb is decreased in the heparinase perfused kidney (*F*) as compared with the control perfusion (*E*) (*A–D*, ×350; *E*, ×400; *F*, ×480).

lected from a panel of mAbs on the basis of their binding to HS. This reactivity was found using crude hybridoma culture supernatants, since we have shown in previous experiments that HS reactivity is due to antibodies complexed to nuclear antigens (14). To evaluate whether these complexed mAbs could bind to the GBM, we performed perfusion studies with complexed and noncomplexed antibody preparations and with reconstituted complexes composed of purified mAb and nucleosomes. The GBM binding was related to the composition of the antibody bound material and the reactivity of the mAb in vitro.

The purification procedures confirm our previous observations that during hybridoma culture nuclear antigens become bound to the mAbs, since we could identify core histones and DNA in the complex bound to the mAb after isolation under physiological conditions. Purification under high salt conditions led to a significant removal of DNA from the bound complex for mAbs No. 2, 32, and 34, whereas after this procedure mAb No. 42 did not contain any nucleosomal material at all. Why, after purification under high salt conditions the DNA content in the complex with mAbs No. 2, 32, and 34 is significantly

Table II Influence of Heparinase Perfusion (Removing HS from the GBM) on the Subsequent Binding of Perfused Complexed mAB No 32

Amount of complexed mAb perfused (μg IgG2a)	GBM staining		Decrease of GBM binding
	HS GAG	HSPG core	
			%
200	–	+	81
300	–	+	76
600	–	+	31

reduced, but the histone content not, is not completely clear It may be related to antigen binding properties of these mAbs, since mAb No 42 is directed against DNA and the other three are directed against nucleosomal epitopes In the case of two of these three mAbs (No 2 and 32) pure antibody was obtained after DNase pretreatment, suggesting that the residual DNA in the complex observed after high salt purification only, plays a role in the binding of the antibody In any case the combined use of DNase pretreatment and high salt conditions yielded noncomplexed mAbs No 2 and 32 In contrast to this, a subfraction of mAb 34 was still bound to histones after this procedure This mAb was further purified using a DNA–cellulose column Complexed antibodies, containing histones, retained on the column and the fraction of non-complexed mAb No 34 was obtained in the effluent and used for further studies

The in vitro analysis of the antigen reactivity of complexed and noncomplexed mAb indicates that binding of nucleosomal material can strongly influence the displayed specificity In general the Farr assay is regarded as the most appropriate test to detect high avidity anti-dsDNA antibodies (22) However, our results show that Farr positivity can be the result of binding of complexes composed of nucleosomal material and anti-nucleosome antibodies, since effective removal of these nuclear antigens leads to disappearance of the binding in the Farr assay This Farr reactivity seems to be the result of the presence of histones within the nucleosomal particle, which is conceivable because of the DNA binding properties of these proteins One could argue that this phenomenon is a peculiar characteristic of these mAbs However, preliminary data (unpublished observations) indicate that this is also true for polyclonal auto-antibodies from SLE patients These observations however, do not reduce the usefulness of the Farr assay in the follow-up of SLE patients, since Farr positivity might be due to the presence in the circulation of auto-antibodies complexed to nucleosomes which we regard as potentially nephritogenic Furthermore, these observations might explain why Farr positivity is associated with onset and/or exacerbation of lupus nephritis (4) and with anti-HS reactivity in SLE patients (31, 32)

The in vitro analysis shows in addition that anti-DNA reactivity both in the Farr assay and in the ELISA and anti-histone reactivity can be a feature of antibodies primarily directed against (neo-) epitopes expressed on nucleosomes The presence of anti-nucleosomal antibodies in SLE has been described before (33–35) In addition, mAbs derived from lupus mice have been described which showed a much higher reactivity towards histone-DNA complexes, than to histones or DNA alone (36, 37), and recently Losman et al described a mAb which only reacted with the intact nucleosome and not with any of the subnucleosomal particles (38) This reactivity is similar to what we observed with purified mAbs No 2 and 32 which also react with a neo-epitope on the intact nucleosome The reactivity of purified mAb No 34 is somewhat different, since it still reacted in non-complexed form with histones From these data it is tempting to speculate that the epitope This mAb recognizes within the nucleosomes is different from that recognized by mAbs No 2 and 32, since these became negative in the histone ELISA once purified Our observations support the possibility that anti-nucleosomal autoantibodies might be present much more frequent in SLE than we appreciate today This is further underlined by the recent report that nucleosomes are a major immunogen for pathogenic auto-antibody inducing T-helper cells (39)

When we tested the various mAb preparation for their capacity to bind to the GBM, we found that the mAbs complexed to nucleosomal antigens (mAbs No 2, 32 and 34) did bind to the GBM, while the noncomplexed, pure mAbs did not bind at all To exclude that additional components from the culture supernatants copurified with the nucleosomal material are responsible for this binding, we prepared nucleosome-antibody complexes by reconstituting pure noncomplexed mAb (procedure III) with highly purified nucleosomes and again saw binding to the GBM The observation that non-complexed mAb did not bind to the GBM does not support a direct binding to intrinsic constituents as has been suggested before (8, 40, 41) When we analyzed the composition of the nucleosomal material which could mediate the binding of mAb to the GBM, we found that the presence of histones was critical Furthermore, a reduction of the amount of DNA within the nucleosome enhanced this binding This observation raises the possibility that partial digestion of the nucleosome by DNase enhances the nephritogenic potential of anti-nuclear antibodies complexed to nucleosomes The involvement of histones in lupus nephritis is substantiated by the recent demonstration of histone deposits in both human and murine lupus nephritis (17, 18, 42) The GBM binding of the complexes containing nucleosomal material used in the present study was different from the binding that we observed previously after subsequent perfusion of histones, DNA and anti DNA In our present study we found a localization throughout the whole width of the GBM and hardly any binding to endothelial or mesangial cells, whereas in our previous experiments (15) the majority of the binding occurred to endothelial cells and the lamina rara interna of the GBM The fact that C3 deposition is induced suggests the nephritogenic potential of this binding Our heparinase perfusion experiments show that HS is an important target for this nucleosome mediated antibody binding, but also that it is not the only anionic ligand within the GBM Additional studies are needed to delineate further these other binding moieties It is tempting to speculate that this nucleosome binding to HS is the explanation for our recent observation that in human (43) as well as in murine lupus nephritis (44) the HS staining in the GBM, as assessed by an anti-HS mAb, has almost completely disappeared, moreover since nucleosomes were able to inhibit the binding of this anti-HS mAb to HS in vitro

In summary, we found that nucleosomal material can mediate the binding of antibodies to the GBM and that complexes composed of nucleosomal antigens and anti-nucleosome antibodies may show anti-DNA reactivity, even in a Farr-type assay which previously was thought to specifically detect high avidity

antibodies to DNA only Binding of these nucleosome containing complexes to the GBM, might be a first step, leading to lupus nephritis

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CHAPTER 5

Nucleosomes and histones are present in glomerular deposits in human lupus nephritis.

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ABSTRACT

Recently, we showed that anti-nuclear auto-antibodies complexed to nucleosomes can bind to heparan sulfate (HS) in the glomerular basement membrane (GBM) via interaction of the histone part of the nucleosome (J.Clin.Invest. 1994;94:568). Histones have been identified in glomerular deposits in human and murine lupus nephritis, and a decreased HS staining was found in glomeruli of albuminuric MRL/l lupus mice, most likely due to masking by deposition of antibodies complexed to nucleosomes (Am.J.Pathol. 1995;146:753). In this study we questioned firstly whether nucleosomes could be identified in glomerular deposits in human lupus nephritis and secondly whether the presence of histones or nucleosomes was correlated with absence of HS staining. Eleven SLE patients with diffuse proliferative glomerulonephritis (DPGN, WHO class IV), six SLE patients with membranous glomerulonephritis (MGN, WHO class V) and nine non-SLE controls (4 with mesangiocapillary glomerulonephritis (MCGN) and 5 with idiopathic membranous glomerulonephritis) were selected. All sections were stained for histones (using a polyclonal rabbit antiserum against peptide 1-21 of histone H3), and with monoclonal antibodies (mAbs) for DNA (mAb #42), histones (mAbs KM2 and #34), nucleosomes (mAbs LG8-1 and LG10-1), IgG, HS (JM 403) and heparan sulfate proteoglycan (HSPG) core protein (JM 72). Using the polyclonal anti-H3 1-21 antiserum, histones were detected in all patients with DPGN and in 2 out of 6 patients with SLE-MGN. Using the anti-histone mAbs, histones were stained in 6 patients with DPGN and in 2 patients with MGN. Nucleosomes were detected in 5 patients with DPGN and in none of the MGN patients. The high avidity anti-dsDNA mAb did not stain any glomerular deposits. HS staining was nearly absent in DPGN, whereas staining was only moderately reduced in patients with MGN ($p=0.001$, Mann Whitney U). Staining of HSPG core protein was normal in all patients.

In conclusion, using the polyclonal anti-H3 antiserum, histones were identified in all patients with DPGN and their presence was associated with a decrease of HS staining. Nucleosomes were identified in 5 out of 11 patients with DPGN. In all other patients nucleosomes were not detected. This is the first demonstration of nucleosomes in glomerular deposits in SLE nephritis. It remains to be elucidated whether the nucleosomal particle deposited in these lesions consists mainly of histones or that nucleosome specific epitopes are masked by anti-nucleosome and/or anti-dsDNA auto-antibodies already bound *in vivo* to these epitopes.

INTRODUCTION

In systemic lupus erythematosus (SLE) about 50% of patients develop renal disease (1). Anti-nuclear antibodies and more specifically anti-dsDNA antibodies are a hallmark for the disease (2). Since often a rise in titer of anti-dsDNA antibodies is found preceding a renal exacerbation (3,4) and renal eluates of patients with lupus nephritis are enriched for anti-dsDNA antibodies (3,5), these antibodies are thought to play a pathogenic role in the initiation of the glomerular disease. Some years ago we showed that certain anti-dsDNA antibodies are able to bind to heparan sulfate (HS), an intrinsic constituent of the glomerular basement membrane (GBM). (6). In subsequent experiments we showed that this HS cross-reactivity was a property of antibodies complexed to nucleosomal antigens (i.e. DNA and histones) (7) and that anti-nucleosome antibodies complexed to nucleosomal antigens were able to bind to HS in the GBM, whereas pure antibodies did not bind (8).

In the meantime it was shown that histones are present in immune deposits of both human (9) and murine (10) lupus nephritis, whereas in murine lupus an association between histone deposits and albuminuria was found (10).

Recently, we found that in human lupus nephritis in the majority of patients HS staining was absent, whereas staining for the heparan sulfate proteoglycan (HSPG) core protein was intact (11). In subsequent studies in MRL/l mice we found that HS staining disappeared in albuminuric mice, and that there was a correlation between HS staining and albuminuria as well as between HS staining and GBM Ig deposits. Since glomerular HS content was not decreased in mice in which HS staining was absent, we concluded that HS might have been masked by nucleosome containing immune deposits (12). Although other investigators showed histones (9,10) or DNA (13) in the deposits, nucleosomes were never identified in glomerular immune deposits.

In the present study we questioned firstly whether nucleosomes were present in the glomerular immune deposits in human lupus nephritis and secondly whether presence of histones or nucleosomes correlated with loss of HS staining. Since we were interested in the interaction between immune deposits and HS in the GBM, patients with diffuse proliferative glomerulonephritis (DGPN) and with membranous glomerulonephritis (MGN) were selected.

MATERIAL AND METHODS

Renal biopsies of SLE patients

Eleven renal biopsies of SLE patients with DPGN and six of SLE patients with MGN were selected. All patients fulfilled at least 4 ARA criteria for SLE. From all renal biopsies 2 μm cryostat sections were cut. In control experiments renal biopsies of 4 patients with non-SLE mesangiocapillary glomerulonephritis and 5 patients with non-SLE MGN were stained.

Immunohistology

All sections were stained in direct IF for IgG deposits and in indirect IF for heparan sulfate (HS), heparan sulfate proteoglycan (HSPG) core protein, histones, DNA and nucleosomes.

Human IgG: Depositions of human IgG were studied in direct IF by incubating sections with FITC labeled $F(ab)_2$ rabbit anti-human IgG (CLB, Amsterdam, the Netherlands) 5 mg/ml diluted 1:400 in PBS containing 1% (wt/vol) BSA (PBS/BSA) for 30 minutes at room temperature. After the staining procedure, the sections were embedded in Aquamount (BDH Ltd. Poole, UK) and examined with a Zeiss fluorescence microscope.

HS and HSPG core protein: HS was stained by incubating the sections with a mouse anti-rat HS monoclonal antibody (mAb, JM 403) that only recognizes HS in basement membranes and cross-reacts with human HS (14). HSPG core protein was stained by incubating with a mouse monoclonal anti-human HSPG-core protein (JM-72, (14)). HS and HSPG-core protein were stained simultaneously on the same slide using double-staining as was described before (14).

Histones: Histones were stained by incubating the sections with a polyclonal rabbit antiserum raised against the N-terminal 1-21 peptide of histone H3 (15) and by incubating with two mAbs against histones derived from lupus prone mice (mAbs KM2 and #34). MAb KM2 is directed against H2A and H4. It is equally reactive with the nucleosome as with a mixture of the five histones (data not shown). MAb #34 shows stronger reactivity with the nucleosome than with a mixture of histones (8). It binds to histone H3 (data not shown). The IF using the polyclonal rabbit antiserum was performed as was described before (9,10). The IF using the mAbs was performed by incubating the sections with mAb KM2 and mAb #34 (10 μg/ml) in

PBS/BSA for 30 minutes at room temperature. Next, sections were incubated with FITC labeled $F(ab)_2$ sheep anti-mouse Ig (Cappel, Organon Teknika, Turnhout, Belgium) 10 mg/ml, diluted 1:750 in PBS/BSA for 30 minutes at room temperature. After the staining procedure, the sections were embedded in aquamount (BDH Ltd. Poole, UK) and examined with a Zeiss fluorescence microscope.

DNA and nucleosomes: DNA and nucleosomes were stained by incubating the sections with mAbs derived from lupus prone mice. The anti-dsDNA mAb (#42) was derived from a panel of anti-dsDNA mAbs described previously (16). The anti-nucleosome mAbs (LG8-1 and LG10-1) only recognize the intact nucleosome and not DNA or histones separately as was described before (17). For IF the sections were incubated with mAb #42, mAb LG8-1 and mAb LG10-1 (10 μg/ml) in PBS/BSA for 30 minutes at room temperature. Further procedures were identical to the IF using the mAbs KM2 and mAb #34.

We also performed a double staining for IgG and nucleosomes using FITC-labeled $F(ab)_2$ rabbit anti-human IgG followed by anti-nucleosome mAb LG 10-1 with TRITC-labeled anti-mouse IgG2b as secundary antibody to compare the localization of the IgG and nucleosome deposits.

Staining of histones using the polyclonal rabbit antiserum and HS-staining was scored semiquantitatively on a 0-8 scale on coded sections by three independent investigators.

Statistical analysis

Presence of histones was compared between kidney biopsies of patients with DPGN and MGN using the Fisher exact test.

HS staining (scored semiquantitatively on a 0-8 scale) was compared between kidney biopsies of patients with DPGN and MGN using the Mann Whitney U test. The correlation between HS staining and staining of histones with the polyclonal rabbit antiserum was tested in Spearman's correlation test.

RESULTS

Staining of IgG, histones, nucleosomes and DNA in glomerular deposits

Using the anti-H3 (1-21) peptide antiserum, in all kidney biopsies of patients with DPGN (Fig 1) and in 2 out of 6 patients with SLE-MGN histones were detected ($p<0.01$)(Table 1). Histone deposits were abundantly present in the walls of the

Table 1. Immunofluorescence findings in renal biopsies of SLE patients with either diffuse proliferative glomerulonephritis (DPGN; WHO class IV) or membranous glomerulonephritis (MGN; WHO class V) and non-SLE controls with either mesangiocapillary glomerulonephritis (MCGN) or idiopathic MGN.

	SLE nephritis		non-SLE nephritis	
Antibodies	DPGN class IV	MGN class V	MCGN	MGN
mAbs[1]				
anti-nucleosome	5/11[3]	0/6	0/4	0/5
anti-dsDNA	0/11	0/6	0/4	0/5
anti-histone	6/11	2/6	0/4	0/5
polyclonal[2]				
anti-H3(1-21)	11/11[4]	2/6	1/4	0/5

[1] *staining of nucleosomes in glomerular deposits using mAbs LG8-1 or LG10-1; staining of histones using mAbs KM2 or #34; staining of DNA using mAb #42.*

[2] *staining of histones using a polyclonal rabbit anti-H3 1-21 serum.*

[3] *number of positive biopsies / number of biopsies tested.*

[4] *$P<0.01$ (Fisher exact test) for SLE-MGN compared to DPGN.*

capillary loops and grossly followed the pattern of IgG deposition (data not shown). Using the anti-histone mAbs in 6 kidney biopsies of DPGN patients and in 2 of the MGN patients histones were identified in the immune deposits (Table 1). MAb #34 stained histones in 5 renal biopsies of DPGN and in 2 biopsies of MGN patients (the same two biopsies which were positive when stained with anti (1-21) H3 serum), and mAb KM2 stained deposits in 3 biopsies of DPGN patients. Nucleosomes were identified in immune deposits of 5 renal biopsies of patients with DPGN (Fig 2) and in none of the biopsies of SLE-MGN (Table 1). MAb LG8-1 stained deposits in 2 renal biopsies of DPGN patients and mAb LG10-1 stained deposits in 4 biopsies of DPGN patients. In the IF analysis with the anti-histone or anti-nucleosome mAbs only limited regions of staining were found in the walls of capillary loops, where the

staining was both focal and segmental. In all biopsies with positive staining, this staining was weak compared to the histone staining using the polyclonal rabbit antiserum in the same biopsies. Anti-dsDNA antibodies did not stain deposits in any of the biopsies (Fig 3). As assessed by double staining, Ig deposits were often observed without staining of nucleosomes but when present the localization of the nucleosome deposits was the same as the IgG deposition. Nucleosome deposits were never found where IgG deposits were absent (data not shown).
In the control experiments (non-SLE biopsies) not one of the mAbs stained glomerular deposits, whereas the polyclonal rabbit anti-H3 (1-21) serum stained glomerular immune deposits in one patient with non-SLE mesangiocapillary glomerulonephritis (Table 1).

Staining of glomerular HS and HSPG core protein

Staining of HSPG core protein was normal in all kidney biopsies studied (Fig 4A, C and E). GBM-HS staining was normal (Fig 4B) or only moderately reduced (Fig 4D) in renal biopsies of patients with SLE-MGN, whereas staining was nearly absent in all patients with DPGN ($p=0.001$), (Fig 4F, Table 1). There was a significant inverse correlation between HS staining and histone deposition as found by the polyclonal rabbit anti-H3 1-21 antiserum ($r_s=-0.77$, $p<0.0001$, Fig 5).

DISCUSSION

In recent years evidence has emerged that nucleosomes may play an important role in the initiation of lupus nephritis (18). Histones have a high affinity for the GBM (19). They are positively charged (pI=10) and probably interact with the negatively charged HS in the GBM. We showed that after subsequent renal perfusion in the rat of histones, DNA and anti-dsDNA mAbs, these antibodies bind to the GBM, whereas subsequent perfusion of DNA and anti-dsDNA leads to limited mesangial binding of antibody, while anti-dsDNA antibodies perfused alone do not bind at all (20). An interaction with HS in the GBM was confirmed by the finding that anti-dsDNA antibodies complexed to DNA and histones were able to bind HS in ELISA (7,8). Subsequently, we showed in the same rat kidney perfusion model that anti-nucleosome antibodies complexed to nucleosomes are able to bind to the GBM. When HS was removed from the GBM by prior perfusion with the HS degrading

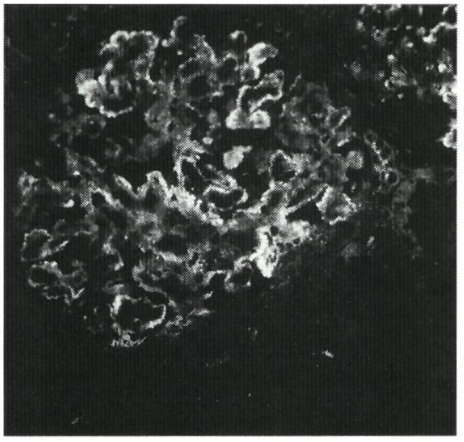


Figure 1: Representative example of a kidney biopsy of a SLE patient with DPGN stained with a polyclonal rabbit anti-H3 1-21 serum, showing abundant histone deposits in the walls of the glomerular capillary loops (x 240).

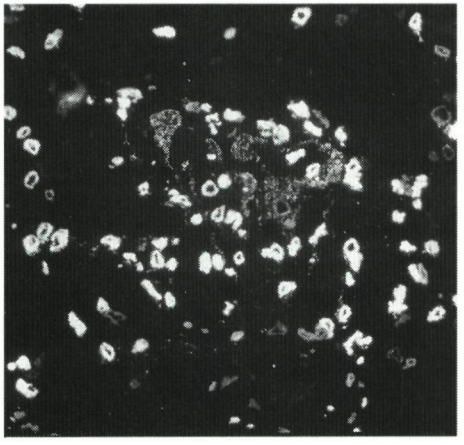


Figure 2: Representative example of a kidney biopsy of a SLE patient with DPGN, stained with a mouse anti-nucleosome mAb (LG10-1). Besides prominent nuclear staining, also segmental staining of the capillary loops can be seen (x 160).

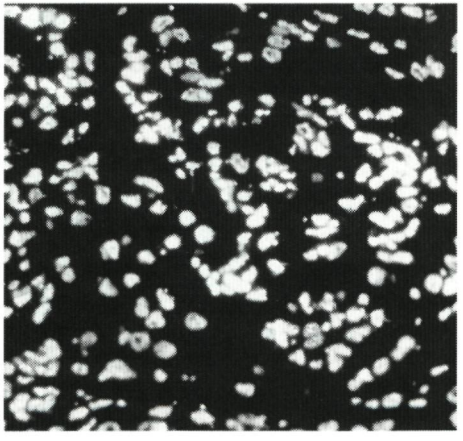
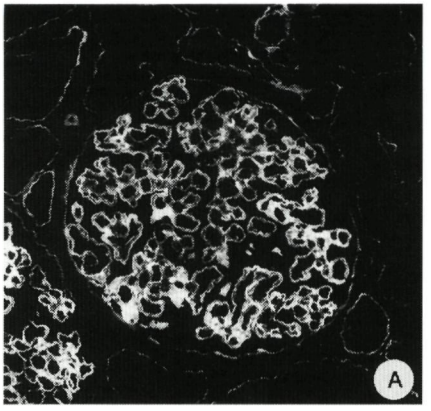


Figure 3: Representative example of a kidney biopsy of a SLE patient with MGN, stained with a mouse anti-dsDNA mAb (#42). Only nuclei stain and no staining in the walls of the capillary loops nor the mesangium is observed (x 240).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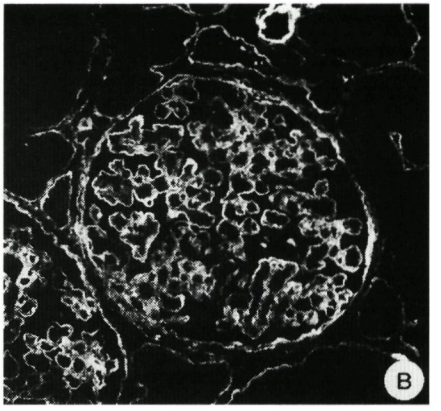
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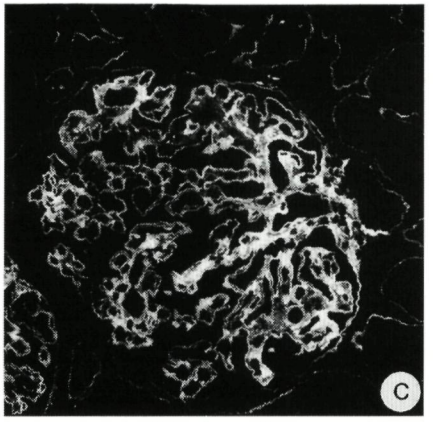
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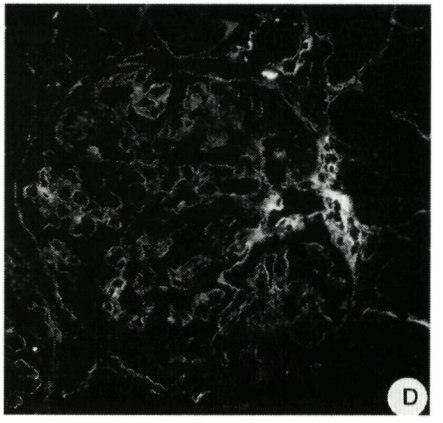
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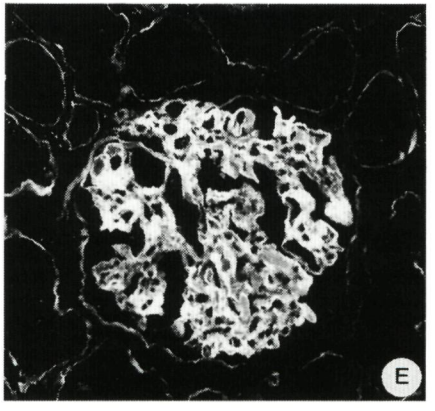
E


F

Figure 4 (opposite page): Representative examples of double staining of a kidney biopsy of two patients with MGN (Fig 4A and 4B, and Fig 4C and 4D) and of a patient with DPGN (Fig 4E and 4F), with the mouse anti-HS mAb JM-403 and with the mouse anti-HSPG core protein mAb JM-72. In Fig 4A, 4C and 4E the biopsies are stained with JM-72, showing intact staining of the HSPG-core protein. In Fig 4B, 4D and 4F the same biopsies are stained with JM-403, showing normal (4B) and moderately reduced (4D) GBM-HS staining in the MGN patients and almost absent GBM-HS staining in the patient with DPGN (4F). (4A and 4B x 200, 4C, D, E and F x 280).

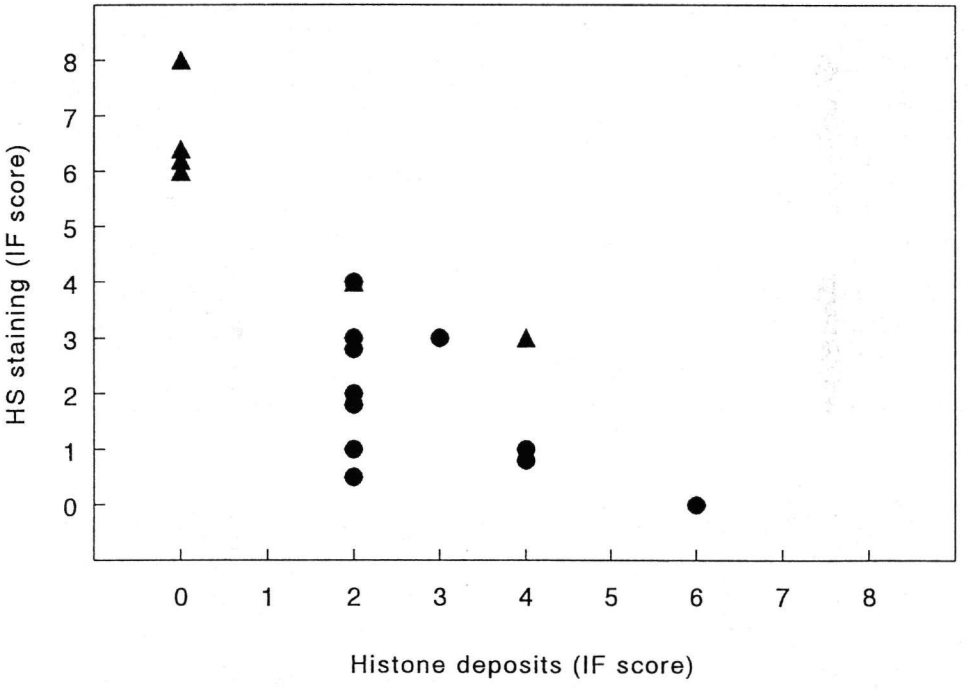

Figure 5: Correlation between HS-staining and staining of histones (as assessed with the anti-H3(1-21) serum) in the walls of the capillary loops. Staining was scored on a 0-8 scale. DPGN (•). SLE-MGN (▲).

enzyme heparinase, the binding of subsequently perfused complexed anti-nucleosome antibodies was greatly reduced (8). Taken together, these experiments show that HS in the GBM is an important ligand for the binding of these nucleosome complexed antibodies and that histones in these complex interact with HS presumably based on charge.

Although histones have been found in the serum of humans (21), it is not very likely that they exist as separate molecules devoid of DNA. In the nucleus, histones are bound to DNA, within the nucleosome. These nucleosomes have been found in the circulation of lupus patients (22). It is assumed that they are released by dying cells. Apoptosis of cells leads to release of oligonucleosomes (23,24), whereas necrosis yields less well defined histone/DNA complexes. It is presently unknown what happens with nuclear material after release in the circulation. DNase and proteinases are present in the plasma so remodelling of the particle is very well possible.

In the present study we analyzed whether nuclear material was present in glomerular deposits in human lupus in more detail by staining for histones, DNA and nucleosomes. Using the polyclonal anti-H3 1-21 antiserum deposits were found in all kidney biopsies of patients with DPGN and in 2 out of 6 patients with MGN. Using the anti-histone mAbs in 6 renal biopsies of patients with DPGN and in 2 MGN patients histone deposits were found, and this staining was considerably weaker than the staining seen with the polyclonal anti-histone antiserum. Because of the broader anti-histone specificity, the polyclonal antiserum has a greater sensitivity for the detection of these histone deposits. Nucleosomes were found in 5 out of 11 kidney biopsies of DPGN patients and in none of the MGN patients. The anti-dsDNA antibodies did not stain any deposits.

These findings suggest that the nuclear particle which is deposited, contains at least histones. Localization of auto-antibodies in the GBM can arise along two different mechanisms. First, they can deposit from the circulation as complexes. Localization via this mechanism leads mainly to subendothelial deposits, as is found mainly in DPGN. The other possibility is an in situ immune complex formation in which the antibody reacts with an intrinsic GBM antigen or an exogenous planted antigen. This mechanism mainly leads to subepithelial deposits as seen mainly in MGN. It is unlikely that the nucleosome binds to the GBM in free non-complexed form and acts as a planted antigen, since renal perfusion of intact nucleosomes mainly leads to mesangial and not to GBM localization (25). This is due to the pI of the native nucleosome which is about 7. Furthermore, the negatively charged DNA is located on the outside of the nucleosomes which reduces the possibility that native

nucleosomes interact with the negatively charged GBM. Such a direct binding can only be envisaged if nuclear material is remodelled by plasma DNase and proteinases which creates a nuclear particle with less DNA and relatively more histones. Such a particle is theoretically able to bind to the GBM leading to in situ-immune complex formation. This mechanism could explain the higher frequency of histone deposits as we found. However, an argument against this possibility is that these histone deposits should have preferentially be present in SLE-MGN, which was not the case. Another, more likely, possibility is that DNA or nucleosome specific epitopes of the nucleosome are covered by anti-dsDNA or nucleosome specific antibodies. When nucleosomes are released in the circulation in lupus patients they will bind to these antibodies and form nucleosome-antibody complexes. Only when these antibodies cover the negatively charged DNA in the nucleosome, the complex is able to bind to the GBM. In this view the overall pI and thus the nephritogenicity of the complex is determined by the antigen specificity of the bound antibody. This view is confirmed by the fact that glomerular eluates are greatly enriched for anti-dsDNA (5,26) and anti-nucleosome antibodies (unpublished observations). In contrast, anti-histone antibodies would cover the histone part of the nucleosome, thereby reducing the positive charge which prevents it from binding to the GBM. Very interestingly, in drug induced lupus, in which anti-histone antibodies are abundantly present and anti-dsDNA antibodies are absent, nephritis is seldom seen (27).

Furthermore, in our experience, glomerular eluates of lupus mice contain only small quantities of anti-histone antibodies which show no correlation with albuminuria (unpublished observations). This mechanism of *in vivo* covering of nucleosome and DNA specific epitopes with a relative sparing of histone specific epitopes could explain the low frequency of nucleosome specific staining and the high incidence of histone specific staining that we observed. Formal proof for this assumption could have come from elution studies on kidney sections, which could lead to uncovering of these nucleosome specific epitopes. However, attempts to achieve this were for technical reasons unsuccesful as we have described previously (12).

The second aim of our study was to find out whether loss of GBM-HS staining in lupus nephritis was associated with histone or nucleosome deposition. In a recent study we found a decrease of glomerular HS staining without loss of HS content. *In-vitro* experiments showed that histones, nucleosomes and anti-nucleosome antibodies complexed to nucleosomes were all able to mask HS in ELISA (12). In the present study this *in vitro* finding was extended since the loss of HS staining correlated with histone deposition. Very interestingly, histone deposition was only found in 2 out of

6 kidney biopsies of SLE-MGN patients, and in these patients HS staining was not or only moderately reduced. This may be due to quantitative differences in glomerular IgG deposition between DPGN and MGN patients, since in DPGN heavier IgG deposits are present. This is in line with our observation in murine lupus nephritis, where an inverse correlation between IgG deposition and GBM-HS staining was found (12). On the other hand it is very well possible that other antibody specificities like anti-laminin or anti-DPPIV (28) play a role in the membranous form of lupus nephritis and that the histopathological differences between DPGN and MGN are based on differences in nephritogenic antibody populations. However, this hypothesis is largely speculative and should be tested by further experiments.
In conclusion, we show for the first time that nucleosomes can be identified in immune deposits of some lupus patients with DPGN. Histones are mainly found in lupus patients with DPGN and their presence correlates with a loss of GBM-HS staining, which is probably due to masking of HS.

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CHAPTER 6

Anti-nucleosome antibodies complexed to nucleosomal antigens bind to cell surfaces and are internalized. Fixation related migration of antibodies leads to *in-vivo* ANA.

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ABSTRACT

It has been suggested that binding of anti-dsDNA antibodies to cell surfaces, followed by internalization and nuclear binding (so called *in-vivo* ANA) is of pathophysiological significance for tissue damage in systemic lupus erythematosus (SLE). We have shown before that pathogenic anti-nuclear antibodies complexed to nucleosomal antigens can bind to heparan sulfate in the glomerular basement membrane *in-vivo*. Since nucleosomes are also reported to bind to the cell surface, we hypothesized that *in-vivo* ANA is a property of anti-nuclear antibodies bound to nucleosomal antigens. Therefore, we studied three monoclonal anti-nucleosome antibodies (mAbs) that exhibit *in-vivo* ANA as seen by immunofluorescence (IF) in mice inoculated intraperitoneally (ip) with the hybridoma producing the mAb. The same mAbs complexed to nucleosomal antigens after intravenous injection into mice induced *in-vivo* ANA, in contrast to purified non-complexed mAbs. To study this in more detail, we incubated complexed mAbs with various cell lines and found binding to the cell surface and subsequent internalization into cytoplasmic vesicles. However, no binding to the nucleus was observed by immuno electron microscopy (IEM) and by confocal laser microscopy. Non-complexed mAbs did not bind to the cell surface. Next, from mice bearing the hybridomas producing the mAbs ip, a small part of the kidney was snap frozen in liquid N_2, fixed with acetone and studied in IF, whereas the remaining part of the kidney was *in-vivo* fixed by renal perfusion with a mixture of 0.01 M sodium periodate, 0.075 M lysine HCl, 0.0375 M Na_2HPO_4 and 2% paraformaldehyde (PLP) and studied in both IF and IEM. In the acetone fixed kidney sections obtained without *in-vivo* fixation we again observed *in-vivo* ANA. However, after *in-vivo* PLP perfusion fixation, no nuclear binding was found. In IEM, localization in cytoplasmic vesicles was seen. In conclusion, anti-nucleosome antibodies complexed to nucleosomal antigens can bind to the cell surface and are transported into the cytoplasm, but do not bind to the nucleus. The reported nuclear localization of anti-nuclear antibodies is due to the type of fixation procedure.

INTRODUCTION

Systemic lupus erythematosus is an auto-immune disease, which affects many organs in the body. Anti-nuclear autoantibodies are the hallmark of the disease, especially anti-double stranded DNA (dsDNA) antibodies (1). These anti-dsDNA antibodies are thought to be responsible for most of the tissue damage in this disease, although the mechanisms by which these lesions are produced in the various organs is still a matter of debate. It has been proposed that anti-dsDNA antibodies are able to bind to cell surface bound DNA (2) or, alternatively, directly via cross reactivity with cell surface proteins (3). This binding may lead to cell damage, for instance it was recently described that it leads to enhancement of apoptosis of rat mesangial cells (4). Other reports mentioned that cell surface bound anti-dsDNA antibodies were internalized and that these antibodies are subsequently transferred to the nucleus leading to derangement of cell functions (5,6). However, one of the first authors who described the binding of anti-dsDNA antibodies to cell surface proteins (7), later discovered that this binding was caused via the intermediary action of nucleosomes or DNA/histone complexes bound to the antibodies (8). Others found that nucleosomes or DNA/histone complexes could bind to cell surfaces, probably via a DNA receptor inducing cytokine production (9). At this moment, it is still unclear whether the binding of nuclear material to cell surfaces, alone or complexed to antibodies, is a charge related phenomenon or mediated via a specific receptor. In favor of a charge dependent interaction is our finding that anti-nucleosome antibodies complexed to nucleosomal antigens are able to bind to the glomerular basement membrane (GBM) *in-vivo*, through an interaction with the negatively charged heparan sulfate in the GBM (10).

When we inoculated in naive mice the hybridomas producing the mAbs that showed this GBM binding, we invariably observed *in-vivo* ANA in kidney sections by immunofluorescence (IF). This nuclear staining can be frequently observed in sections of kidneys from SLE patients or lupus prone mice, and is sometimes explained as a smearing artefact occurring during sectioning of the tissue. Others state that this nuclear staining is the result of an active process *in-vivo* (5,6). In the present study we tested the hypothesis that *in-vivo* ANA is produced by antibodies complexed to nucleosomal antigens. In this perspective the complexed auto-antibody binds to the cell surface via nucleosomal material and, after binding, the complex is internalized. We also analyzed whether this internalization ultimately leads to nuclear binding.

MATERIALS AND METHODS

Preparation and characterization of pure non-complexed antibodies and antibodies complexed to nucleosomal material

Three monoclonal anti-nucleosome antibodies (clones #2, #32 and #34) derived from lupus mice were used. By purification on a protein A sepharose column under physiological conditions (procedure I), high salt conditions (procedure II) or DNase treatment followed by high salt conditions (and in the case of mAb #34 additional separation of histone bound mAb on a DNA-cellulose column) (procedure III), antibodies complexed to nucleosomal material (procedure I and II) or pure non-complexed antibodies (procedure III) were obtained as described before (10).
Purified and complexed antibodies were tested in the Farr assay and in ELISAs (anti-dsDNA, anti-histone and anti-nucleosome) as described before (10). Composition and antigen reactivities of the antibody preparations used have been published before (10) and are summarized in Table 1.
MAbs #2 and #32 are anti-nucleosome mAbs, which in purified form (purification procedure III) only recognize the intact nucleosome and not its components histones or DNA. In pure form (procedure III) mAb #34 also recognizes histones, but its reactivity with the intact nucleosome is stronger. All three mAbs only show DNA reactivity when they are complexed to nucleosomal antigens (purification procedures I and II).

In-vivo studies

Hybridoma inoculation. Hybridoma cells producing the mAbs (5 x 10^6 cells per mouse) were inoculated intraperitoneally (ip) in 8 week old BALB/c mice (n=3 per mAb), which were primed 8 days before with pristane. Two days after development of ascites the animals were sacrificed, the kidneys were flushed with PBS and snap frozen in liquid N_2.
In an additional experiment only a small part of the kidney was snap frozen in liquid N_2 after flushing with PBS, and the remainder of the kidney was perfused during 10 min with a mixture of 0.01 M sodium periodate, 0.075 M lysine HCl, 0.0375 M Na_2HPO_4 and 2% paraformaldehyde (PLP). This was done in order to obtain *in-vivo* fixation. Thereafter, the kidney was cut into small pieces (5x5x1 mm) and immersed in PLP for another 3 h. A small part of tissue was used for IF, while the remaining

Table 1: Composition of bound complexes (panel A), reactivity in Farr assay and various ELISAs (panel B) of the different mAb preparations obtained after purification under physiological conditions (I), high salt conditions (II) or DNase treatment followed by high salt conditions (and in the case of mAb #34 additional separation of histone bound mAb on a DNA-cellulose column) (III).

mAb	#2			#32			#34		
procedure	I	II	III	I	II	III	I	II	III
A: COMPOSITION									
histones*	+	+	−	++	++	−	++	+++	−
DNA‡	+	±	−	++	±	−	+	±	−
B: In-vitro REACTIVITY									
Farr§	6	25	3	70	75	3	17	57	3
ELISA‖									
DNA	178	227	36	98	100	1	178	200	16
histone	55	90	<1	4500	187	1	1777	800	494
nucleosome	3500	4800	3000	5000	7600	5400	7500	12000	12000

** = histone content as assessed on SDS-PAGE; the intensity of the histone staining was scored semiquantitatively by comparing the intensity of the staining with that of the heavy and light chain. ‡ = presence of DNA fragments (of predominately 120 bp) as assessed after labelling with ^{32}P. § = % precipitation of labelled DNA per 12 µg of IgG; values ≤ 3% are regarded as negative. ‖ = titer/mg IgG. (Reproduced with permission from J Clin Invest 1994;94:568-577).*

pieces were used for immuno electron microscopy (IEM). To this end they were washed in PBS for 30 min, cryoprotected by immersion in 80% wt/vol sucrose in PBS for 1 h and snap frozen in liquid N_2.

Intravenous injection. Complexed and non-complexed mAbs (1 mg Ig in 500 µl phosphate buffered saline (PBS)) were injected intravenously (iv) in 6-8 week old BALB/c mice. After 5 or 45 min kidneys were flushed with PBS, taken out and snap frozen in liquid N_2.

Immunohistology of kidney tissue

Direct IF was performed on 2 µm cryostat sections. Sections were fixed for 5 min in cold acetone and air dried. Next, sections were incubated for 1 h at room temperature with $F(ab)_2$ FITC-labeled sheep anti-mouse IgG (Cappel, Organon Teknika NV, Turnhout, Belgium) diluted 1:750 in PBS/1% wt/vol bovine serum albumin (BSA), and 4% normal rat serum. After washing, the sections were embedded in Aquamount (BDH Ltd, Poole, England) and examined with a Zeiss fluorescence microscope.

Double labeling with 4,6-Diamidino-2-phenylindole (DAPI, Sigma, St Louis, MO, USA) was performed by diluting DAPI (1 µg/ml) in Aquamount.

For immuno electron microscopy (IEM), twenty µm sections were incubated with a peroxidase labeled rabbit anti-mouse Ig diluted 1:20 (Dakopatt, Copenhagen, Denmark) and washed three times with PBS. Subsequently, the sections were preincubated in diaminobenzidine medium (0.05% diaminobenzidine wt/vol in TRIS) for 10-15 min, followed by diaminobenzidine medium with addition of H_20_2 to a final concentration of 0.03% vol/vol. The sections were washed in distilled water, postfixed in 0.1 M phosphate buffered 1% wt/vol OsO_4 for 30 min at room temperature, dehydrated, and embedded in Epon 812. Thin sections were prepared on a LKB ultratome, and examined unstained in an electron microscope (Jeol 1200 EX2, Jeol Europe BV).

Binding of complexed and non-complexed mAb to surfaces of cells in culture

For these experiments 3 cell lines were used. CEM and PEER are human T cell lines, that are Fc receptor negative. HL60 is a human myeloid cell line, which bears Fc receptors I and II. Cells were cultured in RPMI 1640, dutch modification (ICN, Costa Mesa, CA), supplemented with 2 mM glutamine and 1mM pyruvate containing

gentamycin (40 μg/ml) and 5% (vol/vol) fetal calf serum (Life Technologies, Gaithersburg, MD). Only cultures less than 3 week old with a viability of >95% as assessed with trypan blue, were used. Cells in suspension (100 μl, $5x10^6$ cells/ml in PBS/1%BSA) were incubated with complexed mAb, non-complexed mAb or an irrelevant mouse IgG2a mAb (100 μl, 200 μg/ml in PBS) for 30 min at 4^0C. After washing twice with PBS/1%BSA they were incubated with FITC conjugated $F(ab)_2$ sheep anti-mouse IgG (100 μl, diluted 1:300 in PBS/1%BSA) (Cappel, Organon Teknika) for 30 min at 4^0C. After washing the cells again twice with PBS/1%BSA the fluorescence intensity was determined with a Fluorescence Activated Cell Sorter (Coulter Electronics Ltd, Luton, UK).

Internalization and ultrastructural intracellular localization of bound mAb

PEER Cells in suspension (1300 μl; 5 x 10^6 cells/ml) were incubated with pure non-complexed or complexed mAbs for 30 min, 2, 5 or 18 h in RPMI containing 5% fetal calf serum at 37^0C. After incubation the cells were fixed in 2% paraformaldehyde (PF)/0.1 M phosphate buffer (PB) pH 7.4 for 1 h at room temperature. The cell suspension was divided in two parts. One part was used for IF and the other part of the suspension for IEM.

In addition a pulse chase experiment was performed. Complexed mAb was incubated at 4^0C with cells (1300 μl; 5 x 10^6 cells/ml) in RPMI containing 10% FCS. Cells were washed three times in PBS containing 1% BSA in order to remove unbound mAb. Next cells were incubated in RPMI containing 10% FCS at 37^0C and studied by confocal laser microscopy at different time points (5 min, 30 min, 2, 5 and 18 h).

Immunofluorescence. The cells were spun down in O.C.T. Tissue-Tek compound (Miles, Elkhart, IN, USA) and snap frozen in liquid N_2. 6 μm sections were cut in a cryostat (-Micron) at -20^0C, picked up on silan coated slides at -20^0C and air dried. After a brief rinse in PBS/0.05% Tween (PBST), the sections were incubated with 1% normal goat serum/PBST for 30 min to block non-specific protein binding. After removal of the latter solution, the sections were incubated for 1 h in a moist chamber at room temperature with goat anti-mouse FITC (Cappel, Organon Teknika) diluted 1:80 in PBST. Then the slides were rinsed with three changes of PBST for 10 min each and mounted with Mowiol (Hoechst, Frankfurt am Main, Germany) /2.5% NaN_3. The sections were viewed with a Zeiss fluorescence microscope.

Immuno electron microscopy. The cells were spun down in 10% gelatin/0.1 M phosphate buffer pH 7.4 (PB), and postfixed in 2% paraformaldehyde (PF)/0.1 M PB

overnight at 4°C. The next day they were cut in small blocks, infiltrated with 2.3 M sucrose, placed on copper stubs and snap frozen in liquid N_2. Ultrathin cryosections (eighty nm) were cut with a cryo-ultramicrotome (Leitz ultracut-FCS), picked up with 2.3 M sucrose and placed on formvar coated copper grids. After blocking the non-specific protein binding with PBS/0.15% glycine/0.1% BSA/0.1% gelatin pH 7.4 (PBSG) for 30 min the sections were incubated overnight at 4°C in a moist chamber with rabbit-anti-mouse IgG (Cappel) 1:500/PBSG. The next day the grids were rinsed three times with PBSG and incubated with protein-A gold 10 nm/1% BSA/PBS for 1 h at room temperature. After rinsing again three times in 0.1 M PBS and 3 times in milli-H_2O the grids were placed on drops of a methylcellulose/uranylacetate (9:1) solution for 10 min on ice and picked up with stainless steel loops. Excess methylcellulose was removed by filter paper and the grids were air dried. The sections were viewed with a JEOL 1010 electron microscope.

Confocal laser microscopy. After fixing with 1% PF/0.1 M PB the cells were spread on silan coated slides and air dried. The cells were permeabilized with 0.5% saponine/0.1 M PB for 30 min and incubated as described for the immunofluorescence technique. After mounting with Mowiol/2.5% NaN_3 the cells were viewed with the confocal laser microscope (MRC 600, Biorad, Hemel Hempstead Herts, UK).

RESULTS

In-vivo ANA after ip hybridoma inoculation in mice

BALB/c mice (n=3 per clone) were injected ip with 5 x 10^6 hybridoma cells. In kidney sections of mice bearing either of the three hybridomas ip a consistent *in-vivo* ANA was observed in IF (Fig 1). DAPI double labeling revealed that all nuclei were ANA positive (data not shown). As we have shown previously with *in-vivo* renal perfusion of nucleosome complexed anti-nucleosomal mAbs (10) we observed also in this experimental set-up glomerular binding (Fig 1).

In-vivo ANA of complexed and non-complexed antibodies after iv injection

To evaluate whether this nuclear binding was, as we hypothesized, due to antibodies complexed to nucleosomes, we injected complexed antibodies iv in mice. At 5 min again binding to glomerular capillary loops and some *in-vivo* ANA was found by IF

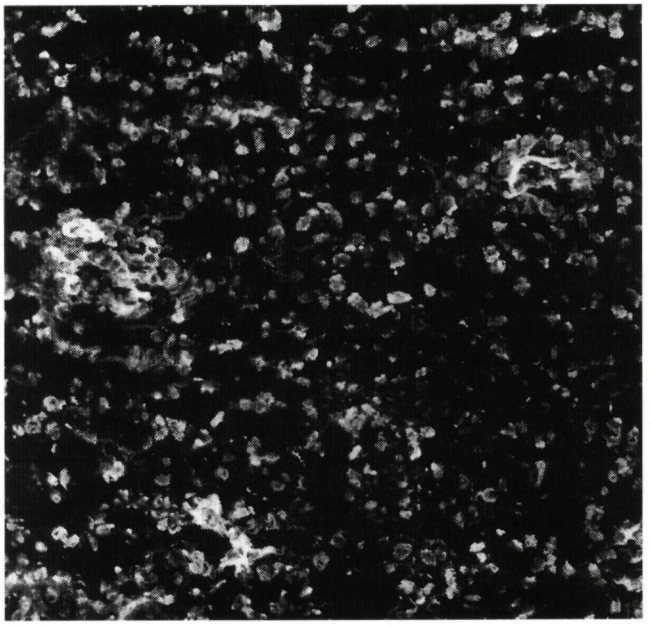

Figure 1: Direct immunofluorescence of kidneys of mice inoculated ip with the hybridoma producing mAb #32 showing in-vivo ANA. Also glomerular deposits are observed (x 240).

(Fig 2A) while after iv injection of pure non-complexed mAb, no glomerular binding and a very faint *in-vivo* ANA was observed (Fig 2B). When kidneys were studied 45 min after iv injection of complexed anti-nucleosomal mAbs the antibodies were found in the cell nuclei by IF (Fig 2C). Double labeling with DAPI revealed that the nuclei of all cells were positive (data not shown). When purified non-complexed mAbs were injected, only a very faint staining of nuclei, similar as shown in Fig 2B, was observed after 45 min. So, nuclear binding was a property of nucleosome complexed antibodies.

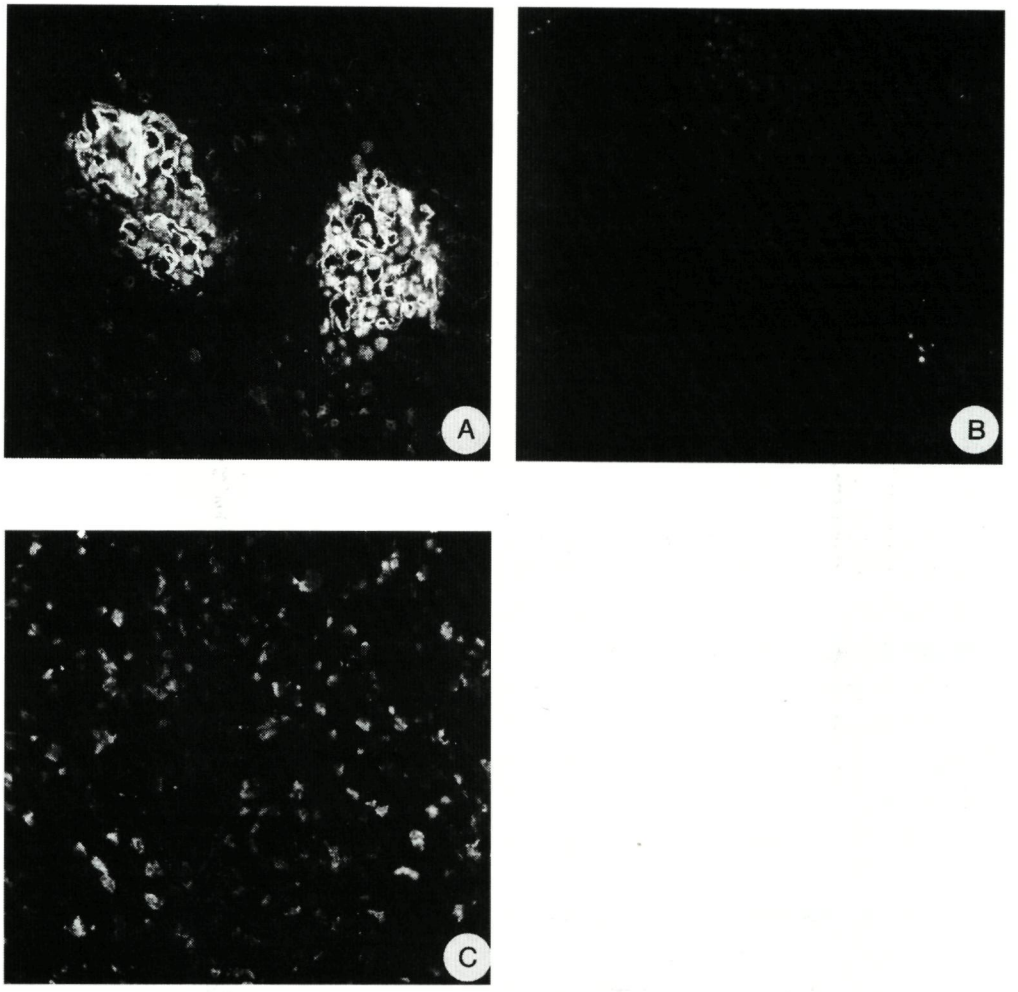

Figure 2: Direct immunofluorescence of mouse kidneys after iv injection of pure antibodies (mAb #32) or antibodies complexed to nucleosomal antigens. When complexed antibodies are injected GBM binding and some in-vivo ANA is observed after 5 min (2A x 240) and after 45 min only marked nuclear binding (in-vivo ANA) is found (2C x 320). When purified mAbs are injected at 5 (Fig 2B x 320) and 45 min no glomerular binding and very faint in-vivo ANA is observed.

Binding of complexed and non-complexed mAb to surfaces of cultured cells

To analyze the course of events leading to *in-vivo* ANA, we turned to binding studies using well defined cell lines. Non-complexed anti-nucleosome mAbs showed hardly any binding to the cell surface of CEM, PEER or HL60 cells (Table 2). However, when the antibodies were complexed to histones and DNA a marked binding was observed. In most cases binding of complexed antibodies containing less DNA (obtained by procedure II) was higher, than that of complexes of the same mAb, obtained by procedure I, which contained considerably more DNA. Cell surface binding was seen with all three cell lines studied, irrespective of the presence of Fc receptors, since CEM and PEER are Fc receptor negative. Fluorescence intensity observed after incubation of control IgG2a mAbs in the same concentration did not exceed background levels (Table 2) except for mAb WT1, which was positive on CEM and PEER cells, since these cells express the CD7 antigen recognized by WT1.

Internalization after binding of complexed anti-nucleosome mAbs to cultured cells

To study whether cell surface binding of antibodies complexed to nucleosomes leads to nuclear binding, complexed mAbs were incubated with cells. Internalization of antibodies was found after two hours, increasing with longer incubation times (5 and 18 h). Antibodies were localized in cytoplasmic vesicles (Fig 3). In the pulse chase experiment after initial binding to the cell surface (Fig 4A), the antibody rapidly appeared in the cytoplasm (Fig 4B). After 18 h of incubation capping of residual mAb was observed. In none of these experiments, repeated several times, nuclear binding of internalized mAbs in living cells was seen.

Effects of fixation on localization of mAb *in-vivo*

Since in the previous experiments, where no nuclear binding was observed, fixation was carried out with paraformaldehyde, we questioned whether the *in-vivo* ANA was related to the fixation technique. To this end we analyzed in a separate experiment the effect of the different fixation procedures carried out on the same kidney. After ip administration of the hybridoma in mice, kidney tissue was fixed in parallel either by freezing in liquid N_2, subsequent cutting of sections and acetone fixation or by initial *in-vivo* fixation with PLP and subsequent processing of tissue. After

Table 2: Binding of complexed and non-complexed mAb to cell surfaces of CEM, PEER and HL60 cells.

mAb	purification procedure#	presence of histones*	presence of DNA‡	CEM	PEER	HL60
–				5	6	6
2	III	–	–	22	30	17
2	II	+	±	117	356	143
2	I	+	+	88	128	55
32	III	–	–	16	34	75
32	II	++	±	166	381	600
32	I	++	++	54	38	ND
34	III	–	–	16	25	81
34	II	+++	±	269	326	529
34	I	++	+	94	101	154
WT1[a]		–	–	171	401	34
1210[b]		–	–	4	5	ND

*The binding of complexed and non-complexed mAb on cell surfaces of 3 cell lines. The binding is expressed as mean fluorescence intensity. Data are derived from a representative experiment. # = for description of purification procedures see Table I. * = histone content as assessed on SDS-PAGE ‡ = presence of DNA fragments as assessed after labelling with ^{32}P (see text).*

a) WT1 is an IgG2a mouse mAb towards human CD7, which is expressed on CEM and PEER cells, but not on HL60. b) 1210 is an IgG2a mouse mAb against human HLA class II, which is not expressed on CEM and PEER. ND = not determined.

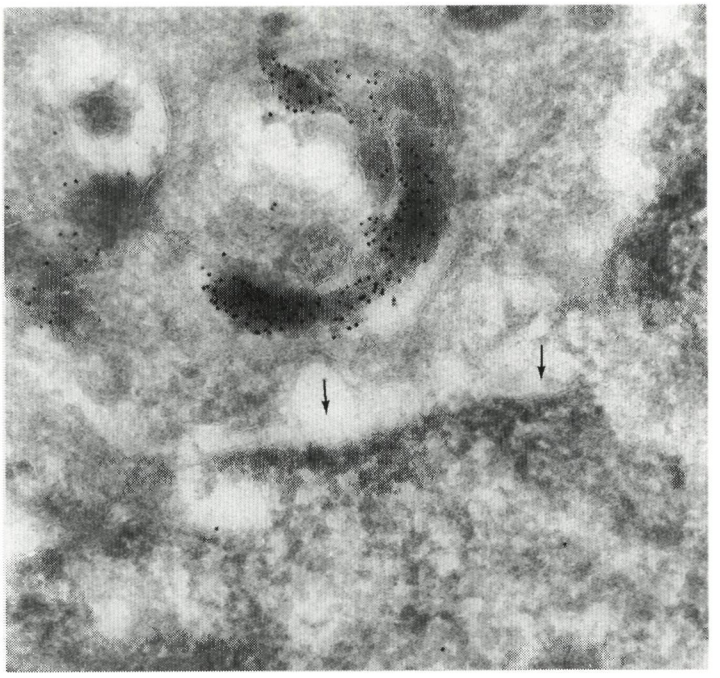

Figure 3: Immuno electron microscopy of lymphoid cells incubated with antibodies (mAb #32) complexed to nucleosomal material. After 5 h gold particles, indicating antibody localization, can be found in cytoplasmic vesicles. The nuclear rim is indicated by arrows. No nuclear binding is observed (x 50000).

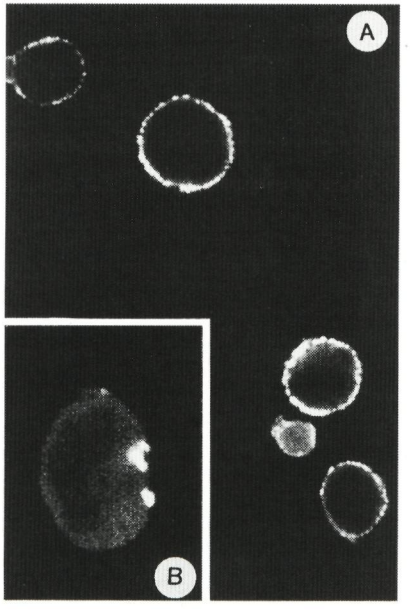

Figure 4: Incubation of antibodies (mAb #32) complexed to nucleosomal material with lymphoid cells. By confocal laser microscopy in a pulse chase experiment after initial binding to the cell surface (4A x 1200), the antibody rapidly appears in the cytoplasm (insert, 4B x 2400). Most importantly these studies did not reveal any nuclear binding in living cells.

cryopreservation and fixation with acetone in IF *in-vivo* ANA was observed identical to that shown in Fig 1. After fixation of the remaining part of the same kidney by perfusion with and immersion in PLP, no nuclear binding was found in this material by IF (data not shown), while by IEM the mAbs were localized in cytoplasmic vesicles (Fig 5), comparable to the localization found in the *in-vitro* experiments with living cells using confocal laser microscopy.

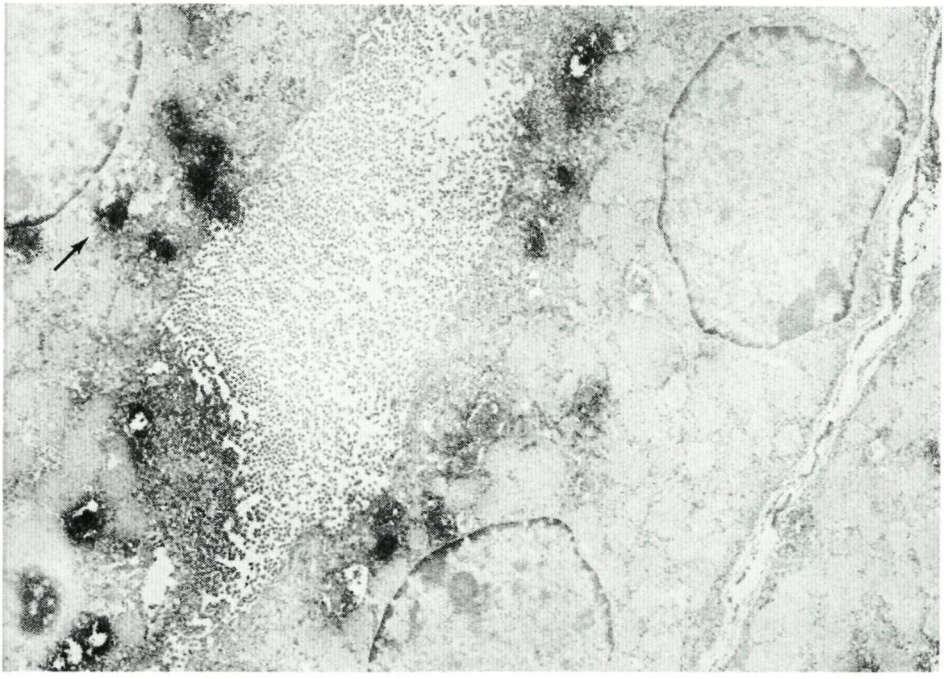

Figure 5: Influence of fixation procedure on in-vivo ANA. A part of the same kidney used for IF in Fig 1, was fixed by in-vivo perfusion and subsequent immersion in PLP. By IEM no nuclear binding is observed. Antibodies are localized in cytoplasmic vesicles, one of which is indicated by an arrow (x 4800).

DISCUSSION

In-vivo ANA (*in-vivo* binding of auto-antibodies to the cell nucleus) as examined by IF has been described by numerous authors, in tissue sections of both patients with SLE (11-13) or other auto-immune diseases (14-16). Recently, it was reported that certain anti-dsDNA mAbs injected into experimental animals reproducibly show

intranuclear localization, whereas others are deposited extracellularly within glomeruli and/or blood vessels (5). This intranuclear localization of anti-dsDNA mAbs was a time and ATP dependent phenomenon. Before internalization binding to a cell surface receptor was demonstrated (6). It was claimed that this *in-vivo* nuclear binding had major pathological consequences like decreased cell proliferation, increased protein synthesis (17), glomerular hypercellularity and proteinuria (5). We also observed nuclear binding when the hybridomas producing the anti-nucleosome mAb were inoculated ip in mice. Since in the ascites of these mice nuclear material is released from dying hybridoma cells, immune complexes are formed *in-vivo* (18). So, this *in-vivo* ANA might be a feature of anti-nucleosome antibodies complexed to nuclear antigens. Indeed, when we injected complexed anti-nucleosome antibodies iv into mice after 45 minutes strong *in-vivo* ANA was observed. After 5 minutes GBM binding was found which was similar to what we observed after renal perfusion of these complexed antibodies (10). We found that nuclear binding of mAb was not restricted to the kidney, but was also found in other organs such as liver and skin (data not shown).

These observations provided evidence that *in-vivo* ANA was a feature of antibodies bound to nucleosomal material. Some of the discrepancies in the literature on this subject might therefore be explained by impurities of the antibody preparations containing variable amounts of nucleosomal antigens. This nucleosomal material can easily be copurified with anti-nuclear antibodies (10) and be missed on SDS-PAGE when Coomassie staining is used.

Before *in-vivo* ANA can develop, auto-antibodies have to bind to the cell surface and have to be transported through the cytoplasm to the nucleus. Binding of anti-dsDNA mAbs to the cell surface, has been described before (19) and was attributed to the binding to the so-called "lupus associated membrane protein" (LAMP) (7). Later on, it was shown that this binding was not a direct cross reactivity of the mAb with LAMP, but that it occurred via histones and DNA (8,20). These findings were however, challenged by a more recent paper describing again that anti-dsDNA mAbs could directly bind to cell surfaces (3). However, one of the mAbs utilized in this latter study (PME 77) was used by Dr. Jacob et al to identify that binding occurred via histones and DNA (8). In line with our observations (10), this mAb shows only weak reactivity with DNA, and strong reactivity with nucleosomes (8). So this mAb resembles the anti-nucleosome mAbs used in this study.

We found only very weak binding of pure non-complexed mAbs to cell surfaces while the complexed antibodies bound considerably stronger. For our experiments we

only used fresh cell cultures with a viability of >95%. This is important since cells in culture can be a rich source for nucleosomal material released in the supernatant and may mediate binding of purified Ig. Using cell cultures with a poor viability, we found indeed considerable binding of purified non-complexed mAbs (data not shown).

The binding to cell surfaces that we observed of histones and DNA is in line with findings of others, showing binding of nucleosomes to cell surfaces, maybe via a DNA receptor (9). It is also possible that binding of complexed antibodies is a charge related phenomenon. This view is supported by our finding that complexed antibodies harboring less DNA bind better to the negatively charged cell surface. When we incubated complexed antibodies with cells we found cytoplasmic localization of mAb, but did not observe any nuclear binding despite 18 h of incubation. Therefore, we returned to the *in-vivo* experiments and found that in the same kidney nuclear binding can be detected when the tissue is processed conventionally for IF, whereas no nuclear localization is found when *in-vivo* PLP fixation is used. By IEM, localization in cytoplasmic vesicles was observed, resembling the picture found with the confocal laser microscopy in the *in-vitro* experiments. This suggests that, when tissue is processed for conventional IF the procedure leads to disruption of intracellular structures, which allows antibodies, present within the cytoplasm, to diffuse and to bind to the cell nucleus, which can occur after sections are cut before the acetone fixation. Another possibility is that acetone fixation is not sufficient and does not prevent migration of antibodies after the fixation procedure. This nuclear localization of mAb is prevented by PLP fixation. Therefore we had to conclude that *in-vivo* ANA is related to the fixation procedure. A similar phenomenon has been described for the p-ANCA due to antibodies to myeloperoxidase (21).

Whether *in-vivo* ANA as found in SLE patients is due to *in-vivo* binding of auto-antibodies to the nucleus or to a fixation related artefact, is not clear yet. However, our study raises serious doubts whether *in-vivo* ANA exists. It is claimed that *in-vivo* nuclear binding of auto-antibodies can lead to tissue damage (5). The fact that *in-vivo* ANA is often encountered in non-inflamed tissue (22) may be an argument that in these instances nuclear binding is a consequence of the fixation process.

Although our results indicate that *in-vivo* ANA is an artefact, the phenomenon of *in-vivo* ANA is a characteristic of pathogenic anti-nuclear auto-antibodies complexed to nucleosomal antigens. The same complexed auto-antibodies are able to bind to the GBM *in-vivo* and activate complement (10) and are related to onset (23) and exacerbations (24) of nephritis in SLE patients. A similar suggestion has been put

forward by Ohnishi and colleagues, who suggested that pathogenic anti-dsDNA antibodies are capable both of binding to nuclei *in-vivo* and to renal basement membranes via DNA/histone (25). Furthermore, cellular binding or intracytoplasmic uptake of these complexed auto-antibodies may be responsible for the derangement of cellular functions as observed by others like interference with apoptosis (4,26), cellular proliferation (5,17), protein synthesis (5) or proteinuria (17). Finally, our studies indicate that the analysis of *in-vivo* ANA is only valid, if the tissue is fixed properly.

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CHAPTER 7

Specificity of monoclonal anti-nucleosome auto-antibodies derived from lupus mice.

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ABSTRACT

Recently, anti-nucleosome antibodies, which do not bind to DNA or to individual histones have been identified in longitudinal studies in lupus mice. These anti-nucleosome antibodies occur early in spontaneous SLE and are formed prior to other anti-nuclear specificities. However, nucleosomal epitopes are yet to be fully characterized.

We selected a panel of 6 monoclonal anti-nucleosome antibodies (mAbs) (#2, #32, #34, PL2-6, LG8-1 and LG10-1) derived from lupus mice. These mAbs were tested in ELISA on subnucleosome structures and on a panel of 53 histone peptides, covering the entire sequence of the five histones. Two mAbs reacted with one of these peptides, namely peptide 83-100 of H3 (mAb #2) and peptide 18-32 of H3 (mAb #34). Based on the nucleosome and subnucleosome ELISA we identified different recognition patterns. Three mAbs showed the highest reactivity towards the intact nucleosome. For two of them (#32 and LG8-1) the nucleosomal epitope was primarily located on H2A/H2B-DNA, whereas for mAb #34 this primary epitope was located on H3/H4/DNA. Two mAbs (#2 and PL2-6) showed the highest reactivity with H2A/H2B-DNA and one mAb (LG10-1) recognized an epitope on H3/H4-DNA. In the subnucleosome ELISA all but one (mAb #32) recognized more than one epitope including DNA complexed to a variety of cationic molecules. Comparing these reactivities we identified for all mAbs one specific nucleosomal epitope, whereas reactivity with other subnucleosomes was comparable to the reactivity towards DNA complexed with cationic molecules. In inhibition experiments both in ELISA and in immunofluorescence it was found that only one of the mAbs (i.e. PL2-6), recognizing an epitope on H2A/H2B-DNA as primary epitope, could be inhibited by H2A/H2B-DNA in fluid phase. The two mAbs recognizing an epitope on H3/H4-DNA as primary epitope could be inhibited by H3/H4-DNA in fluid phase. From these analyses, we conclude first that for these nucleosome specific mAbs linear histone peptides are not very important epitopes. Second, that these mAbs all recognize different epitopes on both H2A/H2B-DNA and H3/H4-DNA and third that some solid phase H2A/H2B-DNA epitopes are not expressed on fluid phase H2A/H2B-DNA. Our findings suggest that in SLE the nucleosome can act as auto-antigen and that there is no immuno dominant epitope within the nucleosome.

INTRODUCTION

In systemic lupus erythematosus (SLE) the formation of anti-nuclear antibodies is a characteristic feature and anti-double stranded DNA (dsDNA) antibodies are regarded as a serological hallmark (1). These anti-dsDNA antibodies have been assigned an important role in the pathogenesis of disease manifestations (2,3). In addition to antibodies to dsDNA also autoantibodies towards histones are formed (4). Recently, antibodies specific for the nucleosome, which do not bind to the constituents of the nucleosome i.e. DNA or histones have been identified (5,6). It has been suggested that the nucleosome is the major auto-immunogen and instrumental in the development of the whole spectrum of anti-nuclear antibodies (7,8). In longitudinal studies in MRL/lpr and BXSB mice, but not in experimentally induced lupus in GVH mice (9), it was found that anti-nucleosome antibodies appear very early in the disease, before the development of anti-dsDNA or anti-histone antibodies. Therefore, it was even postulated that in SLE the loss of tolerance for specific nucleosomal epitopes precedes the formation of anti-dsDNA and anti-histone antibodies (10). In vitro, helper T-cells specific for nucleosomes not only induced anti-nucleosome antibodies but also anti-dsDNA and anti-histone antibodies (7). However, data on the formation of nucleosome specific antibodies in SLE are still scarce. Further characterization of nucleosomal epitopes remains to be accomplished. To address this point we selected a panel of 6 monoclonal anti-nucleosome antibodies (mAbs) derived from lupus mice. By definition the reactivity of these mAbs towards DNA and to individual histones was absent or low compared to their reactivity towards the intact nucleosome. To define their antigenic specificity, these mAbs were tested in ELISA on 4 different subnucleosome structures, which are thought to harbor the epitopes which occur on the intact nucleosome. Furthermore, these mAbs were tested on a panel of 53 histone peptides, covering the entire sequence of the 5 histones. In order to further characterize these nucleosomal epitopes, inhibition experiments both in immunofluorescence (ANA) and in ELISA, were performed. Because we have shown previously (11,12) that the reactivity of monoclonal autoantibodies is strongly influenced by the binding of nuclear constituents to the auto-antibody during hybridoma culture, emphasis was given to the purification of the mAbs, and only purified mAbs were used for the analysis.

MATERIALS AND METHODS

Monoclonal antibodies

Mabs #2, #32, and #34 are from a panel of 60 mAbs, which were obtained by fusion of spleen cells from mice with spontaneous ((NZBxW)F1, MRL/*lpr*) or experimentally induced (parental-F1 chronic graft versus host (GVH) disease) SLE and screened for anti-dsDNA in ELISA (13). After removal of bound nucleosomal antigens these three mAbs lost their anti-dsDNA reactivity and appeared to be nucleosome specific antibodies (12). MAb #2 was derived from a GVH mouse and mAbs #32 and #34 from NZB/W mice. From the same panel mAb #56 was obtained, which is a is a "true" anti-dsDNA antibody since it still bound to dsDNA in purified non-complexed form. Hybridomas of mAbs #2, #32, #34 and #56 were grown in serum free conditions (IMDM, Gibco BRL, Paisley, UK) supplemented with Serum Free Medium Supplement (SF1 (Costar, Cambridge, MA), 2% v/v). Purified non-complexed mAbs #2, #32, #34 and #56 were obtained by purification on a protein A sepharose column (Pharmacia, Uppsala, Sweden) under high salt conditions, after DNase treatment of the culture supernatant (12).
For comparison purposes and further characterization, we have also included 3 previously reported mAbs. PL2-6 is a mAb derived from a MRL+/+ mouse (14). LG8-1 and LG10-1 are mAbs derived from two different MRL/*lpr* mice (15). These mAbs were purified from culture supernatants by affinity chromatography on recombinant protein G-agarose (Pharmacia, Uppsala, Sweden).
The immunoglobulin concentration was determined in ELISA (12). MAbs #2, #32 and #34 belong to the IgG2a and mAbs PL2-6, LG8-1 and LG10-1 to the IgG2b subclass.
The purity of the mAbs (and of the antigens used in the subnucleosome ELISAs which are described below) was checked by SDS-PAGE (8-25%) analysis followed by silver staining (Phast gel system, Pharmacia). The presence of DNA was identified by extraction by phenol-chloroform, labeling with [^{32}P]dATP and analysis on a 6% polyacrylamide gel (Sequagel-6, National Diagnostics, Atlanta, GA) (12).

Tests for antigen specificity of purified mAbs

Purified antibodies were tested in ELISAs using the following antigens: dsDNA, histone, nucleosome, H2A/H2B dimer, H2A/H2B-DNA complex, H3/H4 tetramer and

H3/H4-DNA complex.

- DsDNA, histone and nucleosome ELISA were performed as described before (12).
- H2A/H2B, H2A/H2B-DNA, H3/H4 and H3/H4-DNA ELISAs were performed as described by Losman et al (14) with minor modifications. The individual core histones (purified from calf thymus) were purchased from Boehringer Mannheim. Before use all batches were checked for purity in SDS PAGE as described above and only pure batches were used. H2A/H2B dimers and H3/H4 tetramers were reconstituted by mixing H2A with H2B or H3 with H4 in phosphate buffered saline (PBS) at a final concentration of 1 mg/ml each for 1 hour at 4^0C (16). Next Nunc immunoplates (maxisorp F96, Gibco) were coated overnight at 4^0C with H2A/H2B or H3/H4 in a concentration of 10 µg/ml in 0.1 M glycine, 0.1 M NaCl, 0.1 M NaOH, pH 9. The plates were washed 5 times with PBS/0.05% Tween 20 (PBST) and blocked with 5% fetal calf serum (FCS) in PBS, 150 µl/well, for 1 hour at room temperature. The plates were washed again and samples serially diluted in PBST, containing 5% FCS, were incubated (100 µl/well) for 2 hours at 37^0C. Next, the plates were washed again and 100 µl of peroxidase labeled rat monoclonal anti-mouse Ig antibody (CLB-RM-19, CLB, Amsterdam, the Netherlands) diluted 1:1000 in PBS were added per well. The plates were washed again and developed with 3,5,3',5'-tetramethylbenzidine (Merck), 100 µg/ml in 0.11 M sodium acetate (pH 5.5) containing 0.003% H_2O_2. By adding 2M H_2SO_4, 100 µl/well, color development was stopped after 15 min and the optical density (OD) at 450 nm was measured. When histone-DNA complexes were used as substrate, the ELISA plate was first coated with H2A/H2B or H3/H4 and after washing 5 times with PBS, DNA (Pharmacia) was added for 30 min at 50 µg/ml in PBS at room temperature. Next, plates were washed and further procedures were identical to the anti-H2A/H2B ELISA.
- Protamine/DNA and poly-L-lysine/DNA ELISAs

To analyze whether the reactivity towards DNA was influenced by binding to cations, the reactivity was also measured after precoating the ELISA plates with protamine or poly-L-lysine. Protamine (100 µl, 10 µg/ml) or poly-L-lysine (100 µl, 10 µg/ml) were coated overnight at 4^0C on Nunc Immunoplates. The plates were washed and DNA (100 µl, 50 µg/ml in PBS) was added for 30 minutes at room temperature. The ELISA was further developed as described for the H2A/H2B ELISA.

The titer in all ELISAs was defined as the reciprocal of the dilution giving an OD of 1.0 at 450 nm and expressed per mg of Ig.

- Anti-histone peptide ELISA

This ELISA using 53 synthetic peptides covering the entire sequence of the 4 bovine core histones and of human H1 was performed as described previously (4,17).

Inhibition experiments

Inhibition experiments were performed in ELISA. In these experiments, each mAb was used in a concentration giving about 50% of the maximal OD. Several concentrations of inhibitor were pre-incubated for 20' at room temperature with these mAbs. Next mAb and inhibitor were transferred to a plate coated with the appropriate antigen and the ELISA was performed as described above for the various antigens. In addition, inhibition experiments were performed using nuclear fluorescence in kidney sections. The mAb concentration of the mAbs used was 2 μg/ml because this was the lowest concentration giving maximal nuclear fluorescence. The mAbs were mixed with different concentrations of inhibitor ranging from 0 to 25 μg/ml in two fold dilution steps. After incubation for 20 min at room temperature, mAb and inhibitor were transferred onto kidney sections and indirect immunofluorescence was performed as described before (12). Briefly, 2 μm cryostat sections from BALB/c mouse kidneys were incubated with mAb alone or mAb with inhibitor in PBS containing 1% (wt/vol) BSA (PBS/BSA) for 30 min at room temperature. After rinsing with PBS, the sections were incubated with a F(ab)2 FITC labeled sheep anti mouse IgG (Cappel-Organon Teknika NV, Turnhout, Belgium) diluted 1:750 in PBS/BSA for 30 min at room temperature. After rinsing with PBS, the sections were embedded in Aquamount (BDH Ltd, Poole, England) and examined with a Zeiss fluorescence microscope. Anti-nuclear activity (ANA) was scored by two independent investigators on coded sections and intensity was scored semi-quantitatively on a 0-4 scale.

RESULTS

Specificity of the anti-nucleosome mAbs

Based on the nucleosome and subnucleosome ELISA we identified different recognition patterns (Table 1). Most mAbs showed reactivity with more than one subnucleosome structure, however for all mAbs a primary nucleosomal epitope could be identified. By definition all 6 mAbs reacted with whole nucleosomes, but they

Table 1: Reactivity of the various mAbs to nucleosomes and different subnucleosomal structures.

mAb	#2	#32	#34	LG8-1	LG10-1	PL2-6	WT1[1]
mouse origin	GVH	BWF_1	BWF_1	MRL/l	MRL/l	MRL/n	
nucleosome	3000[2]	**8300**	**12000**	**12800**	12800	19200	<10
DNA	58	<10	<10	455	1000	250	<10
H2A/H2B	<10	<10	80	<10	<10	24000	<10
H2A/H2B-DNA	**5800**	1800	1600	6400	1600	**51200**	<10
H3/H4	<10	<10	1300	<10	<10	<10	<10
H3/H4-DNA	1100	<10	6000	1200	**20500**	10200	<10

[1] *WT1 is a mouse IgG2a anti-human CD7 mAb used as control* [2]*Titer per mg Ig.*

Table 2: Reactivity of mAbs #2 , PL2-6, LG8-1, LG10-1 and #56 (a specific anti-dsDNA mAb) to free non-complexed DNA and to DNA complexed to various positively charged molecules.

	#2	PL2-6	LG8-1	LG10-1	56
DNA	58[1]	250	500	1200	4000
protamine/DNA	364	7500	1400	1600	4000
poly-L-lysine/DNA	364	4000	1500	1200	4000
H2A/H2B-DNA	5800	51200	7200	3600	4000
H3/H4-DNA	1100	10200	1600	40000	4000

[1]*Titer per mg Ig.*

differed in their binding pattern with nucleosome subparticles. MAbs #2 and PL2-6 are anti-H2A/H2B-DNA antibodies since they react better with this antigen than with the whole nucleosome. PL2-6 also shows considerable reactivity with the H2A/H2B dimer. For mAbs #32 and LG8-1, the nucleosomal epitope appears to be primarily located on the H2A/H2B-DNA subparticle, however since these two mAbs reacted better with the whole nucleosome than with H2A/H2B-DNA, additional epitopes seem to be involved. Likewise, LG10-1 has an anti-H3/H4-DNA specificity whereas, for mAb #34 the primary but not exclusive nucleosomal epitope is on the H3/H4-DNA subparticle.

Mabs #2, LG8-1, LG10-1 and PL2-6 hardly bound to DNA, but showed considerable reactivity with both H2A/H2B-DNA and H3/H4-DNA, be it that for each of the mAbs one of these reactivities could be identified as the primary epitope while the other was considerably lower. Since histones are cationic proteins, we questioned whether these antibodies bound to DNA after complexation with a cationic molecule or preferentially to epitopes generated after complexation of DNA to either H2A/H2B or H3/H4, forming its primary epitope. Therefore, we complexed DNA with protamine or poly-L-lysine on the plate, and found an increase in reactivity against cationic complexed DNA compared to DNA alone (Table 2). However, the binding was lower than to the primary histone/DNA epitopes described above. The reactivity of the mAb towards the histone/DNA complex, which was not the primary epitope was comparable to the reactivities found towards DNA complexed to the other cationic molecules. The use of the different precoats did not influence the reactivity of the "true" anti-dsDNA mAb #56, which indicates that the use of cationic precoats did not lead to a different coating of DNA. These experiments indicate that complexation of DNA to a cation can partially mimic but is not identical to the epitope generated after binding of DNA to histones. This suggests that these nucleosome specific mAbs do recognize charge dependent epitopes within the DNA/cation complex molecule but preferentially react with specific epitopes harbored within the histone/DNA complex of the primary epitope. The reactivity of some mAbs towards other DNA complexes than the primary recognized epitope can thus be considered as less specific.

None of the mAbs showed any reactivity when tested in ELISA on individual histones, except for mAb #34 which showed a moderate reactivity towards H3. In the histone peptide ELISA, mAb #2 bound to the 83-100 peptide of H3 and not with any other peptide tested. MAb #34 bound to the 18-32 peptide of H3. However, the OD

Table 3. Binding of mAbs histone to synthetic peptides (2μM). Only results of mAbs #2, #34 and a control mAb WT1 are shown. The other 4 anti-nucleosome mAbs were negative on all histone peptides.

Antigen	#2	#34	WT1
H1 peptides	neg[a]	neg	neg
H2A peptides	neg	neg	neg
H2B peptides	neg	neg	neg
H3			
1-21	0.05	0.06	0.11
18-32	0.00	**0.40**	0.02
30-45	0.00	0.05	0.04
40-55	0.14	0.10	0.08
53-70	0.07	0.04	0.04
68-85	0.08	0.00	0.00
83-100	**0.40**	0.04	0.00
98-112	0.08	0.01	0.01
107-123	0.03	0.04	0.01
111-130	0.09	0.07	0.07
118-135	0.12	0.05	0.04
130-135	0.04	0.01	0.01
H4 peptides	neg	neg	neg

Results are expressed as OD values and positive results (OD ≥ 0.30) are underlined.
[a]neg: negative (OD < 0.30) for all peptides tested.

Table 4: Results of inhibition experiments in ELISA and IF. In both experiments the inhibitor was the antigen which was the primary epitope which the mAb recognized. In ELISA inhibition of binding of the mAb to this primary epitope and in IF inhibition of ANA was tested.

mAb	specificity	ELISA	IF
#2	H2A/H2B-DNA	>50[1]	>25[2]
#32	H2A/H2B-DNA	>50	>25
LG8-1	H2A/H2B-DNA	>50	>25
PL2-6	H2A/H2B-DNA	12.5	2
#34	H3/H4-DNA	1	1
LG10-1	H3/H4-DNA	25	10

[1]The concentration of inhibitor (μg/ml) giving 50% inhibition of OD in ELISA or [2]50% reduction of ANA scored semiquantitatively in IF.

found on these two histone peptides was low. The other mAbs did not bind to histone peptides in this assay. The results of these experiments are given in Table 3.

Inhibition experiments

For all mAbs was tested whether the primary antigen recognized by these mAbs was able to inhibit in fluid phase the binding of these mAbs to the same antigen in solid phase ELISA or to nuclei in IF. The results found in ELISA and in IF were comparable (Table 4). From the mAbs recognizing H2A/H2B-DNA as primary nucleosomal epitope, only for PL2-6 the binding to H2A/H2B-DNA in ELISA and to nuclei in IF was inhibited by H2A/H2B-DNA in fluid phase. For mAbs #2, #32 and LG10-1 fluid phase H2A/H2B-DNA could not inhibit this binding. In contrast, for both mAbs recognizing H3/H4-DNA as primary epitope (i.e. #34 and LG 10-1), this binding could be inhibited by fluid phase H3/H4-DNA. From these experiments we conclude that the nucleosomal H2A/H2B-DNA epitope recognized by mAbs #2, #32 and LG10-1 is not expressed by fluid phase H2A/H2B-DNA and that it is different from the epitope recognized by PL2-6.

DISCUSSION

In recent years more and more evidence has emerged that the nucleosome plays an important role in the development of the auto-antibody repertoire in SLE. Already more than a decade ago, it was found that when rabbits were immunized with chicken erythrocyte nucleosomes, antibodies to the various individual histones were formed, in a striking similar pattern as found in the sera of SLE patients. However, antibodies against dsDNA could not be detected (18). Later it was found, that when New Zealand white rabbits were immunized with RNA-complexed to histones, they developed an autoimmune-like response including antibodies against dsDNA and peptides of the Sm-D antigen (19). In this study it was concluded that altered nucleosome particles, rather than native nucleosomes may represent the antigenic stimulus giving rise to autoantibodies in SLE. Recently, it was shown that not DNA but the nucleosome is the antigen which is recognized by pathogenic auto-antibody inducing T-helper cells (7). It was also found that anti-nucleosome antibodies are seen early in spontaneous murine lupus, before the occurrence of anti-dsDNA and anti-histone antibodies (10,20) and that in human SLE the prevalence of anti-nucleosome antibodies is very high (6,21). However, the study of anti-nucleosome antibodies in polyclonal sera is hampered by the fact that anti-dsDNA and anti-histone antibodies are also able to bind to the nucleosome and will contribute to the total anti-nucleosome reactivity in serum. Analysis of nucleosome specific antibodies in serum or plasma can therefore only be performed after removal of anti-dsDNA and anti-histone antibodies. Using this approach the existence of nucleosome specific auto-antibodies was recently demonstrated in human lupus (22).

The existence of antibodies specific for (sub)nucleosomes not reactive with either DNA or histones separately, was also shown in studies analyzing monoclonal antibodies (5,12,14,23). Until now the fine specificities of these antibodies have not been studied in full detail.

In this study we describe 6 monoclonal anti-nucleosome antibodies, which do not or only weakly bind to DNA or individual histones. When these were tested in ELISA with subnucleosome structures they all appeared to be different. For each mAb a major epitope could be identified, being either H2A/H2B-DNA or H3/H4-DNA on the intact nucleosome (mAbs #32, #34, LG8-1), H2A/H2B-DNA (mAbs #2, PL2-6) or H3/H4-DNA (LG10-1). However, most mAbs recognize more than this major nucleosomal epitope. In experiments using DNA complexed to a variety of cationic molecules we could show that the binding to the other "minor" nucleosomal epitopes

is comparable to the binding to DNA complexed to cationic molecules and can thus be considered as less specific. In contrast to mAbs #2, LG8-1 and LG10-1, mAb PL2-6 also binds to H2A/H2B dimer. This reactivity was first described in patients with procainamide induced lupus (24). For this mAb H2A/H2B-DNA binding can be explained by its reactivity towards H2A/H2B. MAb #34 not only binds to H3/H4-DNA, but also to a lesser extent to H3/H4 and H3.

The finding that DNA complexed with positively charged molecules can mimic nucleosomal epitopes has clinical consequences, since the majority of the routinely performed anti-dsDNA ELISA's use a protamine or poly-L-lysine precoat to catch DNA. These assays therefore may not only detect anti-dsDNA but also anti-nucleosome specificities.

Four out of these six mAbs recognize an epitope located on H2A/H2B-DNA as primary nucleosomal epitope. That H2A/H2B-DNA comprises major epitopes of the nucleosome is underlined by the observation that the prevalence of anti-H2A/H2B-DNA reactivity in human lupus was found to be very high and comparable to anti-chromatin (≈ anti-nucleosome) reactivity (21). Since it was found that anti-nucleosome and anti-H2A/H2B-DNA reactivity was found early in spontaneous murine lupus, before the occurrence of anti-dsDNA antibodies, it has even been postulated that tolerance against H2A/H2B-DNA is lost early in the disease and that reactivities against other epitopes on the nucleosome (like dsDNA and H3/H4-DNA) develop later (10). So it seems that H2A/H2B-DNA is a central and major epitope for lupus auto-antibodies.

Until now little is known about the pathogenic potential of anti-nucleosome antibodies. Recently, we described for mAbs #2, #32 and #34, that when these antibodies are complexed to nucleosomal antigens, they can bind via the histone-part to the GBM *in vivo* and activate complement (12). As we describe in this paper, #2 and #32 recognize an epitope located on H2A/H2B-DNA and #34 on H3/H4-DNA. It was recently reported in human lupus that the presence of anti-H2A/H2B-DNA reactivity correlates with kidney disease (21). In a study in graft versus host mice, levels of antibodies against chromatin core particles decreased significantly before the appearance of proteinuria, suggesting their involvement in glomerular injury (9). On the other hand anti-H2A/H2B-DNA was first described in patients with procainamide induced lupus (25), and in these patients renal disease is very rare (26). It is therefore tempting to speculate that the H2A/H2B-DNA complex harbors different epitopes, which define the nephritogenic potential of the various anti-H2A/H2B-DNA antibodies. In our view (27) antibodies which bind the positive

charges on the N-terminal ends of H2A/H2B will prevent the subsequent binding to the GBM, while antibodies which will neutralize the DNA associated negative charges will render the complex more nephritogenic.
MAb #2 also recognizes the 83-100 peptide of H3 in the histone peptide ELISA. Since binding to H2A/H2B-DNA is stronger than to H3/H4-DNA and the OD found in the histone peptide ELISA is low, these data suggest that this linear epitope is not very important in the binding of the mAb to the nucleosome. A linear epitope (18-32 H3) on H3 was identified for mAb #34 which might explain its reactivity towards H3.
Inhibition experiments in IF and ELISA showed that preincubation of mAbs #2, #32 and LG8-1 with H2A/H2B-DNA did not inhibit ANA in IF nor H2A/H2B-DNA binding in ELISA. We conclude that the nucleosomal epitope recognized by these mAbs is not expressed by H2A/H2B-DNA in fluid phase. It appears therefore that conformational changes which take place during coating are necessary for binding. The fact that most H2A/H2B-DNA reactive antibodies could not be inhibited by fluid phase H2A/H2B-DNA has implications for absorption studies on polyclonal sera with fluid phase H2A/H2B-DNA, which have been performed in the past (25). In view of our results it is possible that not all H2A/H2B-DNA reactive antibodies will be absorbed by fluid phase H2A/H2B-DNA.

In summary, we tested 6 anti-nucleosome mAbs on subnucleosomal structures and histone peptides. Some linear histone epitopes were identified, but these seem to be of minor importance. After detailed analysis, all mAbs appeared to be different. They all recognize different nucleosomal epitopes on both H2A/H2B-DNA and H3/H4-DNA. We conclude that nucleosomes evoke auto-autobodies in lupus which are directed against a multitude of epitopes on the nucleosome. Our findings support the view that in SLE different parts of the nucleosome can act as auto-antigens which trigger the formation of anti-nucleosome antibodies.

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CHAPTER 8

Recognition of the histone H3 peptide 83-100 by monoclonal anti-dsDNA antibodies.

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Antibodies against double stranded DNA (dsDNA) are viewed as the hallmark for systemic lupus erythematosus (SLE) (1). The trigger for the formation of these antibodies, however, has always been puzzling. It is now generally accepted that the auto-antibody response in lupus is antigen driven since somatic mutation and affinity maturation occur and T-cell help is required (2,3). Although immunization of normal mice with bacterial DNA can induce anti-single stranded DNA antibodies, anti-dsDNA antibodies are not formed and in general mammalian DNA is non-immunogenic (4,5). Since antigen presenting cells (APCs) present peptides and not nucleotides after processing of the antigen, DNA itself is an unlikely candidate to evoke the anti-dsDNA response. In the nucleus DNA is closely associated with histones, which have a function in the first steps of packaging the DNA into chromatin and in gene transcription. The nucleosome consists of a histone octamer (two histone H2A/H2B dimers and a histone H3/H4 tetramer), 146 base pairs (bp) of DNA wrapped twice around the octamer (forming the so called core particle), and the linker DNA (between 20 and 60 bp) joining adjacent core particles to form the nucleosomal array. Histone H1 binds outside the core particle to the linker DNA region (6).

The nucleosome has been proposed recently as an important immunogen in lupus, both at the T and B cell level, which may induce the formation of anti-dsDNA antibodies (7). When we tested a series of anti-nuclear antibodies on a panel of histone peptides, we found that not only anti-histone and anti-nucleosome antibodies could bind to certain histone peptides (data not shown), but unexpectedly also anti-dsDNA antibodies. We then raised the question whether more anti-dsDNA antibodies recognized (in addition to DNA) non-DNA nucleosomal epitopes and tested 6 monoclonal anti-dsDNA antibodies (mAbs) for their reactivity towards a series of overlapping synthetic peptides of the five histones H1, H2A, H2B H3 and H4.

The mAbs used in this study were obtained from two different lupus mouse strains, namely mAb #2 from experimentally induced lupus mouse with parental-F1 chronic graft versus host (GVH) disease, and the other five mAbs (#36, #42, #51, #53 and #56) from different NZB/W mice. All selected mAbs belong to the IgG2a subclass except for mAb #51 which carries the IgG2b isotype.

The mAbs were produced, purified and characterized as described previously (8). As it is known that DNA and histones can be copurified with anti-dsDNA antibodies (8), particular care was given to the purification of the mAbs. For the analysis only highly purified mAbs were used, not complexed to nucleosomal antigens, which was checked as described (8). One of these (i.e. mAb #2) showed only weak binding to

Table 1. Binding of mAbs to dsDNA, individual whole histones and synthetic histone peptides.

	Monoclonal antibodies					
	#2	#36	#42	#51	#53	#56
dsDNA	**0.81**	**>3.00**	**>3.00**	**>3.00**	**>3.00**	**>3.00**
H1						
peptides	neg[1]	neg	neg	neg	neg	neg
Whole H1	0.12	0.22	0.15	0.17	0.26	0.07
H2A						
peptides	neg	neg	neg	neg	neg	neg
Whole H2A	0.04	0.16	0.21	0.07	0.19	0.12
H2B						
peptides	neg	neg	neg	neg	neg	neg
Whole H2B	0.08	0.10	0.29	0.10	0.13	0.18
H3						
1-21	0.05	0.10	0.24	0.10	0.27	0.10
18-32	0.00	0.04	0.09	0.05	0.14	0.00
30-45	0.00	0.07	0.10	0.06	0.29	0.04
40-55	0.14	0.19	0.29	0.10	0.19	0.28
53-70	0.07	0.14	0.18	0.13	0.23	0.18
68-85	0.08	0.05	0.13	0.02	0.09	0.01

83-100	**0.40**	0.06	**≥3.00**	0.12	**1.45**	**1.94**
91-104	ND	0.10	0.14	0.13	ND	0.13
98-112	0.08	0.05	0.17	0.01	0.04	0.02
107-123	0.03	0.05	0.16	0.02	0.06	0.05
111-130	0.09	0.04	0.25	0.03	0.04	0.12
118-135	0.12	0.06	0.15	0.00	0.05	0.05
130-135	0.04	0.02	0.19	0.00	0.02	0.03
Whole H3	0.06	0.06	0.22	0.04	0.09	0.05
H4						
1-29	0.12	0.27	**0.38**	0.06	0.21	0.29
27-44	0.06	0.12	0.28	0.13	0.27	0.19
42-59	0.08	0.02	0.18	0.00	0.03	0.07
57-74	0.02	0.01	0.19	0.00	0.00	0.00
72-89	0.06	0.03	0.20	0.00	0.02	0.03
85-102	0.05	0.00	0.14	0.00	0.03	0.00
Whole H4	0.06	0.06	0.22	0.05	0.10	0.02

Results are expressed as OD values and positive results (OD≥0.30) are underlined.
[1]neg = negative (OD<0.30) for all peptides tested. ND = not determined.

DNA and a strong reactivity with the intact nucleosome, while the other five mAbs were "true" anti-dsDNA antibodies. In purified form two out of these five antibodies (i.e. mAbs #42 and #56) showed high titers in the Farr assay, which is regarded as the "golden standard" for the detection of anti-dsDNA antibodies. These antibodies were tested for their reactivity in ELISA towards whole histones and towards 53 synthetic histone peptides, covering the entire sequences of the 4 bovine core histones and human H1 (9,10).

None of the six mAbs reacted with any of the whole histones. In contrast, four out of six mAbs bound to one and the same histone peptide, i.e. the H3 83-100 peptide. In addition, mAb #42 recognized the H4 1-29 peptide, although the OD value found in this ELISA was just above the cut-off and low compared to the OD value observed with H3 83-100 (Table 1).

By ELISA inhibition experiments, we were able to show that the binding of mAbs #2, #42, #53 and #56 to dsDNA could be efficiently inhibited by peptide H3 83-100 and vice-versa, although in all experiments DNA was a more powerful inhibitor than H3 83-100 (data not shown). In control experiments, neighboring histone peptides (i.e. H3 91-104 and H3 98-112) could not inhibit the antibody binding to DNA.

As outlined already the nucleosome has been assigned an important role in SLE. Firstly, antibodies exclusively reactive with the intact nucleosome and not with its components DNA or histones were identified (11,12). Secondly, in longitudinal studies in spontaneous lupus mouse models, tolerance for the nucleosomal epitopes seems to be lost first since antibodies to chromatin appear first, while later on anti-dsDNA and anti-histone antibodies are formed (3,13). More importantly, some nucleosome-specific T-helper clones from SNF1 lupus mice were found to be able to induce anti-dsDNA and anti-histone antibodies in vitro (7). It has also been shown that immunization of animals with altered nucleosome particles induced a variety of anti-nuclear antibodies which are also found in patients with SLE (14). Lastly, we have provided evidence that nucleosomes are able to mediate the binding of auto-antibodies to the glomerular basement membrane (8,15).

The observed reactivity towards the H3 83-100 peptide is not an unique property of a single lupus strain, since mAb #2 was derived from a GVH mouse and the others from NZB/W F1 mice.

The finding that these murine anti-dsDNA mAbs bind to H3 83-100 may contribute to the understanding of the nature of the B cell epitope involved in the production of auto-anti-dsDNA antibodies. We postulate that the nucleosomal epitope recognized by these mAbs is composed of both dsDNA and the H3 83-100 peptide, but that the mAbs can also show reactivity against a part of this epitope, i.e. dsDNA and the H3 83-100 peptide. A similar mechanism has been proposed for mAbs binding both H2A/H2B dimer and DNA (16). In this regard, it is interesting to know that native mammalian DNA itself is non-immunogenic, but when it was coupled to certain proteins (Fus 1) an anti-dsDNA response emerges after immunization of normal mice (17).

Although it is rather uncommon that the same peptide of an antigen serves both as

a B- and T-cell epitope, it might be possible that this H3 peptide is generated during processing of the nucleosome by the antigen presenting cell (APC), and that it may be part of the antigen presented to the T-cell. In this view this H3 peptide could act as a cryptic nucleosomal T-cell epitope (18) postulated by Mohan et al (7). Further research is needed to analyse whether this H3 83-100 peptide coupled to DNA of restricted length is indeed recognized by lupus derived T-cells. Preliminary studies have shown that immunization of rabbits with this peptide alone does not evoke an anti-dsDNA response (C Stemmer and S Muller, unpublished data). If further research will indeed identify this peptide as an important part of the antigen inducing anti-dsDNA antibodies, this raises the possibility to interfere with the anti-dsDNA auto-antibody formation with T-cell vaccination using a modified form/presentation of this antigen. A similar approach has been shown to be effective in other auto-immune diseases like adjuvant arthritis (19).

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CHAPTER 9

Apoptosis, nucleosomes, and nephritis in systemic lupus erythematosus.

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Apoptosis, nucleosomes, and nephritis in systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterized by the production of antinuclear autoantibodies. The serological hallmark of SLE is the presence of antibodies against double-stranded DNA. It is generally assumed that these anti-DNA antibodies participate in the development of lesions in this disease. The primary event inducing the formation of anti-DNA antibodies has always been puzzling, since it has been very difficult to demonstrate the presence of free DNA in serum of SLE patients [1]. Furthermore, native DNA is usually considered non-immunogenic [2, 3]. In recent years, several studies have been published which suggest that not DNA itself, but DNA complexed to histones (nucleosomes) is the immunogenic particle involved both in the induction of pathogenic anti-DNA antibodies, and in the pathophysiology of SLE

In eukaryotic cells, DNA is packed in the nucleus in the form of chromatin. In chromatin, adjacent nucleosomes are linked by about 60 bp of DNA. Nucleosomes are the fundamental repeating units of the chromatin string, and are responsible for the compaction of DNA Each nucleosome is built up by eight core histone molecules (forming the histone octamer), one histone H1 molecule and 146 bp of DNA [4], as shown in Figure 1.

Lately, it has become evident that at least in certain murine models of SLE (*lpr* and *gld* mice), and perhaps also in SLE patients as well, the process of apoptosis is aberrant Apoptosis (programmed cell death) results in fragmentation of the nucleus by internucleosomal cleavage of the chromatin. Normally, these apoptotic cells are phagocytosed rapidly, but if this does not occur nucleosomes may be released. On the other hand, apoptosis of maturing T cells is involved in establishing and maintaining tolerance, and therefore a disturbed apoptosis may cause a breakdown of tolerance. These recent data provide elements for a new concept of SLE, both for the induction as well as for the effector phase of the disease. We will first review the evidence for disturbed apoptosis in SLE, and then discuss how this relates to the immune response against nucleosomes. Subsequently, the significance of nucleosomes for the disease manifestations of SLE (with emphasis on glomerulonephritis) will be discussed.

Dysregulation of apoptosis in SLE

The term "apoptosis" was first used in 1972 to describe the process in which cells undergo several morphological changes, including condensation and fragmentation of the nucleus without disruption of the plasma membrane [5] It was later shown that activation of an endogenous nuclease is involved in the internucleosomal cleavage of chromatin [6]. The induction of apoptosis is under strict regulation by a variety of factors, including signals from surrounding cells With respect to T cells, for example, both cytokines (such as interleukin-1, interleukin-2, interferon-γ) and costimulatory molecules (like ICAM-1, VCAM-1, LFA-3 and B7) present on antigen-presenting cells determine whether a T cell is driven to apoptosis or to cell proliferation [7–10]. Once a cell has become apoptotic, it should be removed in a rapid and efficient manner while still intact, to prevent the release of potentially toxic cell contents. This removal of apoptotic cells occurs by phagocytosis. Macrophages are the most important effector cell in this respect, but other cell types like epithelial cells apparently can also ingest neighboring cells undergoing apoptosis. Several different receptors on the phagocyte surface may be involved in this process, such as phosphatidylserine receptor and thrombospondin receptors [11]. The morphology of apoptotic cells in tissues and *in vitro* has been described in detail [12, 13].

It has been very difficult to demonstrate the presence of free DNA in serum from SLE patients, but when DNA was found it occurred in the form of (oligo)nucleosomes [1]. A potential source for these nucleosomes are apoptotic cells, since nucleosomes were spontaneously released from cultured normal murine spleen cells, whereas this process did not occur when apoptosis was blocked with zinc sulfate [14]. Also during culture of human lymphocytes nucleosome release occurred, which was strongly correlated with the degree of lymphocyte apoptosis [15]. It is, however, not precisely known how nucleosomes are released from apoptotic cells. It is conceivable that in some cases these nucleosomes derive from apoptotic bodies. These appear at the cell surface of several cell types during apoptosis as a result of packaging of cellular components as membrane-bounded blebs. After their budding from the cell, apoptotic bodies are usually rapidly ingested by phagocytes. Some of these apoptotic bodies contain chromatin [13] In a recent study on UV-injured keratinocytes, it was demonstrated that some of the apoptotic bodies contained nucleosomes, ribonucleoproteins (that are also targets of autoantibodies in SLE), and in addition fragments of the endoplasmatic reticulum or the nuclear membrane [16]. Nucleosomes may, after their release from apoptotic cells, also adhere to specific "nucleosome receptors" present on the surface of viable cells [17, 18]. In contrast to the programmed process of apoptosis, cell death by necrosis is a more random process of cellular destruction [12, 13] that is not expected to yield intact nucleosomal particles.

In recent years it has become evident that, at least in certain murine models, SLE is directly linked to a defect in apoptosis. We will first discuss the data obtained in lupus-prone and transgenic mice, and then address this question for human SLE. In mice, the *lpr* and *gld* genes provide single gene models of systemic autoimmunity (including the formation of anti-dsDNA antibodies).

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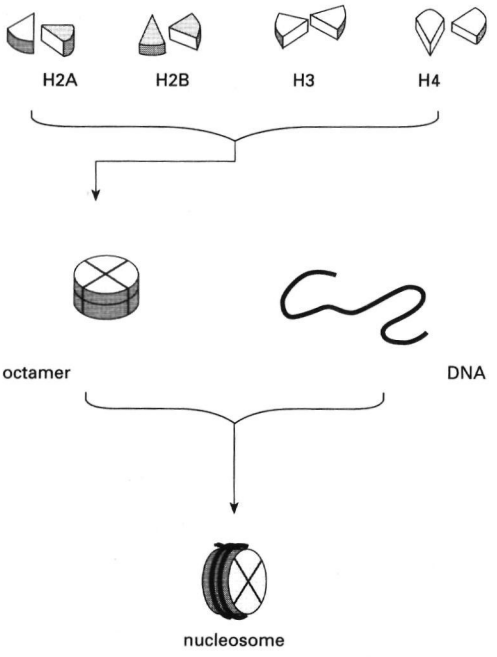

Fig. 1. *Diagram showing how the nucleosome is built up by eight histone proteins (4 homodimers of H2A, H2B, H3, and H4) and two superhelical turns of 146 bp of DNA.* The N-terminal parts of the histone molecules (approximately one third of the total molecule) contain positively charged residues which are located on the outside of the nucleosome. Histone H1, which is located on the outside of the nucleosome, is not shown in this diagram.

Although expression of the *lpr* gene in both B and T cells is required for autoantibody production in *lpr* mice [19, 20], the molecular basis of the *lpr* syndrome became only apparent when Watanabe-Fukunaga et al [21] demonstrated that *lpr* mice lack the expression of the *Fas* antigen. The *Fas* molecule (also known as APO-1, and designated CD95) belongs to the TNF receptor family, and is a cell-surface receptor that mediates apoptosis. The aberrant transcription of the *Fas* gene in *lpr* mice is caused by the insertion of a retroviral sequence in an intron [22–24]. Early replacement of the *lpr* gene by the normal *Fas* gene will correct the accelerated autoimmune disease in these mice [25]. These findings demonstrate that in this model there is a causal relationship between dysregulation of apoptosis and autoimmune disease. The natural ligand for *Fas* has recently been cloned; it is a transmembrane protein and a member of the TNF family [26]. The *gld* mouse strain has a mutation of the *Fas* ligand gene [27]. It therefore appears that either a defect in the receptor (*lpr*) or its ligand (*gld*) causes systemic autoimmunity. How does the defect in the *Fas* antigen cause autoimmunity in *lpr* mice? In view of the important role of apoptosis in shaping the T cell repertoire by negative selection, one is inclined to think that a defective *Fas* antigen would cause aberrant thymic selection. It appears, however, that thymic selection of CD4+ or CD8+ T cells in these mice proceeds normally [28]. In contrast, both subsets of mature T cells exhibit decreased apoptosis in response to antigen stimulation and therefore it has been suggested that autoimmunity in this model results from a failure of peripheral tolerance [29, 30]. Also in *gld* mice, the T cell defect apparently is contained within the compartment of activated, mature T cells rather than in the thymus [31]. It seems probable that the defective *Fas* gene in *lpr* mice may also cause a decreased antigen-induced apoptosis of mature autoimmune B cells [21]. It is important, however, to realize that even in *lpr* mice other genes are involved as well [32]. Since MRL/+ mice (which lack the *lpr* gene) also develop lupus (although to a lesser extent, and later in life), it must be concluded that *lpr* accelerates rather than causes the disease. In other murine models of SLE, such as NZB/W mice, no abnormalities of the *Fas* gene have been detected [22].

Apart from the *Fas* gene, there are several other genes involved in the regulation of apoptosis. There are pathways for the induction of apoptosis which are independent of the *Fas* antigen, such as the TNF receptor [33]. Furthermore, there are genes that decrease the susceptibility for apoptosis, such as the *bcl-2* gene [34]. The *bcl-2* gene was first discovered in B cell lymphomas, which have a permanent high expression of the *bcl-2* protein. When in transgenic mice *bcl-2* was brought under the control of an immunoglobulin promotor, the number of B cells increased, and autoantibodies against nuclear antigens (including histone and DNA) were produced, resulting in immune complex glomerulonephritis [35]. In another study with transgenic mice, it could be demonstrated that *bcl-2* inhibits the clonal deletion of autoreactive B cells in the periphery but not in bone marrow [36]. In *bcl-2* knockout mice, fulminant lymphoid apoptosis was observed, causing disappearance of the lymphoid system within four weeks after birth [37, 38]. Recent studies have revealed that the *bcl-2* gene is a member of an expanding family of similar genes, some of which have the same effect as *bcl-2* (protection against apoptosis), whereas others like *bax* will increase the susceptibility for apoptosis [39]. The *bax* protein can form a heterodimer with the *bcl-2* protein and in this way neutralise the effect of *bcl-2*. The *bcl-2* protein apparently exerts its protective effect by activating an anti-oxidant pathway in the cell [40]. Many stimuli inducing apoptosis are known to generate reactive oxygen species. The relative amounts of *bax* and *bcl-2* in a cell may therefore determine the susceptibility of the cell for apoptosis.

In human SLE, no defects in expression or function of the *Fas* antigen have been found [41]. This does not imply, however, that apoptosis is completely normal in human SLE. Two phenomena have been described that may contribute to disturbed apoptosis in human SLE. First, serum levels of soluble *Fas* were found to be elevated in several SLE patients. Due to alternative splicing, the *Fas* encoded protein can also occur in a soluble form. Soluble *Fas* can inhibit apoptosis, since after injection in normal mice, it causes increased numbers of B cells and autoimmune features [42]. Secondly, in lymphocytes from SLE patients the expression of *bcl-2* is increased [43]. This may be a reflection of *in vivo* activation, since lymphocyte activation is known to increase the transcription of *bcl-2* [44]. Despite these increases of soluble *Fas* and intracellular *bcl-2*, lymphocytes from SLE patients display an increased rate of "spontaneous" apoptosis *in vitro* [15], as do lymphocytes from *lpr* mice [45]. One should be cautious, however, to extrapolate from these *in vitro* findings to the *in vivo* situation.

It has been postulated that lymphocytes from *lpr* mice may be "primed" for apoptosis, but prevented from dying *in vivo*. Once placed in culture *in vitro*, such cells would then rapidly die [45]. With respect to lymphocytes from SLE patients, the protective effect of soluble *Fas* in serum will be diminished or absent when cells are cultured *in vitro*. Furthermore, as mentioned above, the rate of apoptosis of SLE lymphocytes in response to antigenic stimulation is decreased [29, 30] instead of increased. It may be important in this respect to precisely define the subset of lymphocytes that is being studied (double-negative T cells, or CD4-positive T cells, or B cells), and their antigen specificity. It is conceivable that some cells in SLE patients (or lupus-prone mice) display an increased rate of apoptosis whereas for other cells (depending on cell type, activation state, and specificity) apoptosis may proceed at a decreased rate, or at the same rate as in normal individuals. Therefore, although the *Fas* antigen is normal in human SLE, apoptosis appears to be abnormal, as evidenced by an increased concentration of soluble *Fas*, an increased expression of *bcl-2*, and an increased rate of apoptosis when lymphocytes from SLE patients are cultured *in vitro*. It remains to be proven that the abnormalities of apoptosis observed in human SLE are a cause of disease (as appears to be the case in the murine SLE models discussed above) rather than a consequence

Dysregulation of apoptosis can contribute to the pathogenesis of lupus nephritis in two different ways. First, apoptosis appears to be an important mechanism to induce tolerance of T cells towards self-antigens [46, 47]. This implies that a disturbance in apoptosis may cause persistence of autoreactive T cells and in this way affect the magnitude of the anti-nucleosomal immune response. As discussed above, one of the consequences of a defective *Fas* antigen is breakdown of peripheral T cell tolerance. Secondly, autoreactive lymphocytes that have escaped apoptosis in response to autoantigen may be "primed" for apoptosis. Some of these cells may be induced to become apoptotic by other mechanisms (unrelated to *Fas*). Removal of such apoptotic lymphocytes may be less effective (depending on the microenvironment) than when apoptosis occurs by the "normal" (antigen-induced) mechanism. In this respect it may be relevant that within tissues phagocytosis of apoptotic bodies occurs rapidly, but not when they are formed in a fluid medium [13]. A possibility that deserves further investigation is that in SLE patients phagocytosis of apoptotic cells (and apoptotic bodies) might be decreased. Although there are no published data yet on this subject, it has been known for a long time that in SLE there are profound defects in other forms of phagocytosis (such as complement-mediated phagocytosis in lupus-prone mice [48], and Fc receptor-dependent phagocytosis in SLE patients [49]). If phagocytosis of apoptotic cells is indeed defective in SLE, this could also affect the persistence of non-lymphoid apoptotic cells (such as UV-injured keratinocytes [16]).

Nucleosomes as immunogenic particles in SLE

In what way can dysregulation of apoptosis contribute to autoimmunity against nucleosomal antigens in SLE? What is the role of nucleosomes, and how are T lymphocytes (double-negative, or CD4+ cells) and B cells involved in this process? As discussed above, apoptosis is the most likely source of nucleosomes. Lymphocytes from SLE patients demonstrated an increased rate of apoptosis *in vitro*, and the number of apoptotic cells in freshly isolated lymphocytes was also slightly increased [15]. If this reflects the *in vivo* situation, the increased number of apoptotic lymphocytes might lead to the release of increased amounts of nucleosomes. One way that nucleosomes can evoke an immune response is by polyclonal activation of B cells. Both with murine and human lymphocytes, polyclonal B cell activation by nucleosomes has been demonstrated [14, 50]. The secretion of interleukin-6 that was observed after interaction of nucleosomes with murine spleen cells [18] may further enhance this polyclonal B cell activation.

Although polyclonal B cell activation by nucleosomes may occur, there are many arguments in favor of the hypothesis that pathogenic anti-DNA antibodies are the result of an antigen-driven immune response (such as clonal expansion, IgG class switch, somatic mutations). T-helper (Th) cells are required for the production of these anti-DNA autoantibodies. Since there is an expanded population of double-negative T cells (CD3+, CD4−, CD8−) in SLE [51, 52], and because these cells exhibit a preferential use of distinct Vβ genes both in *lpr* mice [51] and in humans [53], these cells might be involved in the initiation of autoimmune disease. Indeed, $\alpha\beta$ CD4−CD8− (as well as $\gamma\delta$ CD4−CD8−) T helper cell lines could be isolated from patients with active lupus nephritis [52, 54]. The large majority, however, of Th cells supplying help for production of pathogenic anti-DNA, are CD4+ cells and not double-negative cells [54]. Furthermore, recent experiments with MHC class II-deficient *lpr* mice have demonstrated an important role for CD4+ Th cells, whereas double-negative T cells did not appear to be essential for initiating disease [55] It was found in lupus-prone mice that only a fraction of autoreactive T cell clones can induce anti-DNA autoantibodies *in vitro*, and accelerate the development of lupus nephritis when transferred *in vivo*. The specificity of these pathogenic Th cells has been studied in some detail Approximately 50% of them were specific for nucleosomal antigens. Importantly, nucleosome-specific CD4+ T cells were not detectable in normal mice, but they were found in the spleens of lupus-prone mice as early as one month of age, long before any autoimmune manifestation. These nucleosome-specific Th cells not only augmented the anti-nucleosome response of syngeneic B cells, but also the anti-histone and anti-DNA response. The importance of nucleosomes as immunogenic particles was underlined by the finding that injection of purified nucleosomes into preautoimmune mice could significantly accelerate the development of glomerulonephritis [56].

An intriguing question in this respect is the nature of the epitope recognized by the pathogenic Th cell. These nucleosome-specific Th clones were not activated by free DNA or histones, the two components of nucleosomes. This result suggests that either nucleosomes are taken up more efficiently by antigen-presenting cells, or that critical Th cell epitopes in the histones are protected from degradation by being bound to DNA [56]. Another possibility is that after antigen processing of nucleosomes, MHC class II molecules present self-peptides that were previously cryptic. The possible significance of such cryptic epitopes for the induction of autoimmunity has recently been reviewed [57] It has also been suggested that the presence of endoplasmatic reticulum or nuclear membrane within the apoptotic bodies causes an increased generation of reactive oxygen species which may induce oxidative modification of autoantigens. The unique peptide fragments generated in this way could then, in genetically susceptible individuals, be presented to Th cells and induce an autoimmune response [16].

Whatever the precise nature of the epitope recognized by the

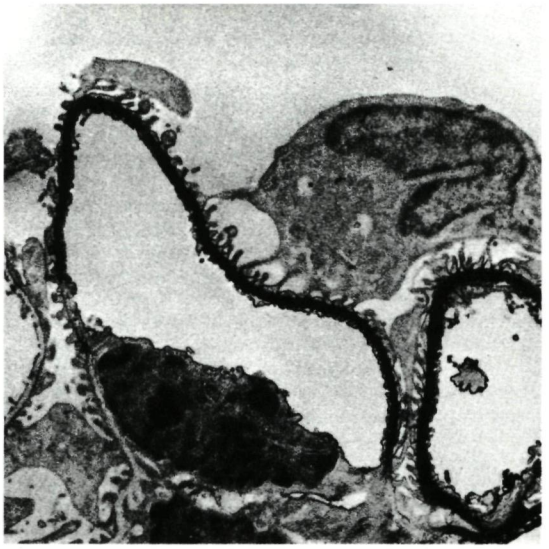

Fig. 2. *Immunoelectron microscopy after perfusion of a monoclonal antibody complexed to nucleosomal antigens.* Throughout the width of the GBM, binding of the complexed antibody is seen (the noncomplexed, pure antibody did not bind at all).

pathogenic Th cells, it is obvious that nucleosome-specific Th cells were only present in lupus-prone and not in normal mice [56]. In normal individuals, apoptosis also occurs but no anti-DNA antibodies are found. Furthermore, injection of nucleosomes into normal mice does not result in formation of anti-DNA autoantibodies or nephritis [58]. It is apparent then that apart from the extracellular presence of nuclear antigens, there must be other factors involved in the induction of such autoreactive T cells. It is well known that susceptibility to SLE is influenced by genes encoded within the MHC complex [2, 55]. Furthermore, in *lpr* mice the virtually absent expression of *Fas* may cause breakdown of (peripheral) tolerance, as discussed above. This may result in the prolonged survival *in vivo* of autoimmune T and B cells.

Anti-nucleosome antibodies

The occurrence of antibodies specific for nucleosomes or subnucleosomal structures in SLE provides further evidence that nucleosomes are immunogenic particles in SLE. These antibodies, which only react with the intact nucleosome or subnucleosomal structures, but not with its components DNA or individual histones, have first been noted in drug induced lupus. In procainamide induced lupus not only antibodies to H2A-H2B complex were found [59], but also to (H2A-H2B)DNA complexes [60]. Further analysis revealed that the antibodies recognize an epitope created by the monomeric, trimolecular histone-DNA complex [61]. Studies in lupus prone mice revealed that antibodies against chromatin (such as nucleosomes) are the result of an antigen driven response and precede the occurrence of anti-DNA or anti-histone antibodies [62]. The authors concluded that anti-DNA antibodies are a subset of the wide spectrum of anti-chromatin (nucleosome) antibodies, which is also favored by the observation that nucleosome specific T helper cells cannot only induce an anti-nucleosome response, but also reactivity towards DNA [56]. Recently, it was shown that the prevalence of anti-nucleosome antibodies is very high (88%) in human lupus [63] and that the titers of anti-nucleosome reactivity correlate with disease severity [64]. One should realize, however, that the study of these reactivities in polyclonal SLE sera is hampered by the fact that anti-DNA and anti-histone antibodies will also bind to nucleosomes. This problem is circumvented if one studies the specificity of monoclonal antibodies derived from lupus prone mice or SLE patients. Indeed, reactivity against histone/DNA complexes has been found in such monoclonal antibodies (mAbs). From MRL/Mp +/+ mice mAbs were obtained which exerted reactivity against the (H2A-H2B)DNA complex [65], and recently a murine mAb directed against the intact nucleosome, which did not recognize any subnucleosomal structure, was described [66]. Also in mAbs derived from SLE patients reactivity against a mixture of DNA and histones has been detected [67]. We recently found that anti-nucleosome antibodies complexed to nucleosomal material exerted anti-DNA reactivity both in ELISA and Farr assay. After removal of the bound nucleosomal material the anti-DNA reactivity disappeared and only anti-nucleosome reactivity remained [68]. This indicates that in SLE sera reactivity towards DNA can be a feature of anti-nucleosome antibodies. In fact, we now have preliminary data that after purification of anti-DNA positive SLE sera, a substantial part of the anti-DNA reactivity is lost, whereas reactivity against other, nominal antigens (for instance anti-EBV) is unaltered. This suggests that the occurrence of anti-nucleosome antibodies in SLE might be more prevalent than is assumed until now.

Relevance of nucleosomes for the pathophysiology of SLE nephritis

Classically, not nucleosomes but DNA has been thought to play a role in the development of tissue lesions in lupus through the formation and subsequent deposition of DNA/anti-DNA immune-complexes [69]. However, formal proof for this assumption is rather lacking. It has been very difficult to detect circulating free DNA or DNA complexed to anti-DNA antibodies [70–72]. Rumore and Steinman [1] showed that DNA in the circulation of SLE patients was present in the form of (oligo)nucleosomes. Based on these observations it is likely that DNA is not present in naked form in the circulation but complexed in (oligo)nucleosomes. In addition, it is difficult to envisage how the anionic charged DNA can have affinity for the anionic charged GBM.

The first notion, that nucleosomes might play a role in the pathophysiology of glomerular disease in lupus, came from experiments with cross reactive anti-DNA antibodies. Working with anti-DNA mAbs which bound to heparan sulfate (HS) we found that this cross reactivity was not exerted by the antibody itself, but was mediated by nucleosomal material complexed to the antibody [73]. HS is an intrinsic constituent of the GBM, and the negatively charged side chain of heparan sulfate proteoglycan. It is responsible for the negative charge of the GBM, and thus for the charge-dependent permselectivity of the GBM [74]. Neutralization of this HS associated charge, or antibody binding to HS leads to albuminuria [75, 76]. We postulated that the positively charged histones within the nucleosomal part of the immune complex

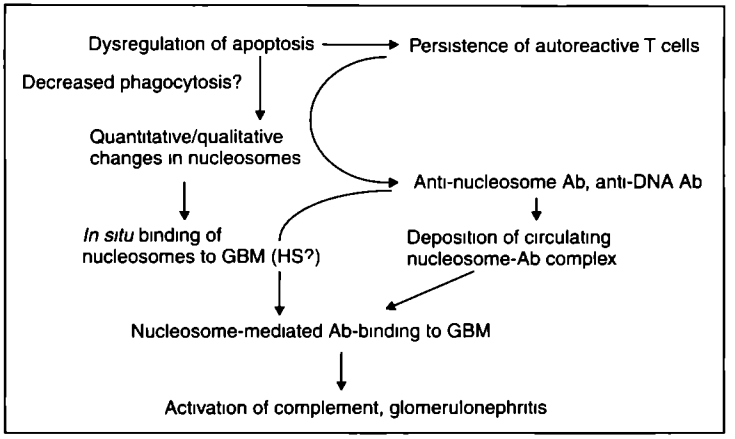

Fig. 3. *Proposed mechanisms linking dysregulation of apoptosis to lupus nephritis* Nucleosomes can be released from apoptotic cells (decreased phagocytosis might be a contributing factor) Persisting nucleosome-specific T cells can provide help for production of anti-nucleosome antibodies Nucleosomes can mediate the binding of such antibodies to anionic structures in the GBM, such as heparan sulfate (HS)

could interact with HS and that this interaction might lead to albuminuria by neutralization of charge or by eliciting an inflammatory reaction [73]. At the same time Schmiedeke et al [77] indeed showed that histone aggregates have a high affinity for the GBM. We subsequently showed in a rat kidney perfusion system that histones can mediate binding of subsequent perfused DNA and anti-DNA and induce subepithelial or subendothelial localization of the antibody depending on the perfusion protocol [78]. We propose that nucleosomes act as planted antigens or as mediators for binding of autoantibodies to the GBM. Although nucleosomes have a pI of about 7, they contain many positively charged N-terminal regions which are located on the outside of the particle It has even been shown that molecules with a net negative charge, but with cationic regions can bind to the GBM [79] Indeed, we could show that nucleosome containing immune complexes are able to bind to the GBM, whereas the purified antibodies do not bind. Binding of such complexes to the GBM is illustrated at the ultrastructural level in Figure 2. The pathogenic potential of this binding was underlined by the fact that complement activation took place. Using heparinase perfusion prior to perfusion of nucleosome containing immune complexes, we could show that the ligand in the GBM was partly but not solely HS [68]. The exact composition of the nucleosomal material in the immune complex which mediates the binding to the GBM *in vivo* is not clear yet. In our studies we obtained immune complexes from hybridoma culture supernatants and found that the four core histones but not H1 were present, and DNA in the complexes were predominantly the size of about 120 bp The presence of antibody bound to the nucleosome seems to be a critical determinant, since we found that perfusion of naked nucleosomes did not lead to a comparable binding to the GBM. Furthermore, the specificity of the bound antibody could be important for the overall pI of the complex, for instance anti-DNA and anti-nucleosome antibodies increase the pI of the complex and thereby the nephritogenicity This concept is supported by the recent finding that pathogenic anti-DNA antibodies, which are able to bind to HS in the GBM via nucleosomes, harbor more charged amino acids (like arginine) than non-pathogenic anti-DNA antibodies [80]. In this concept binding of anti-histone antibodies, by binding to the histone part of the nucleosome would both decrease the pI and the nephritogenicity of the complex This latter hypothesis is in line with the clinical observation that renal disease is rare in SLE-like syndromes characterized by the occurrence of predominantly anti-histone antibodies as in drug induced lupus

What are the data to support the significance of nucleosomes *in vivo*? Since HS reactivity is a feature of immune complexes consisting of anti-nuclear autoantibodies and nucleosomal antigens, anti-HS reactivity in plasma is an indirect way to identify the presence of these immune complexes. Anti-HS reactivity has been found to correlate with renal symptoms in lupus patients [81–83], although anti-HS reactivity was not present in every episode with renal manifestations [83]. Further evidence for the role of these immune complexes comes from our observation that the staining for glomerular HS in both human [84] and murine [85] lupus nephritis is strongly reduced or even absent. Despite a nearly complete loss of HS staining in mice with heavy and prolonged albuminuria, glomerular HS content was unaltered. Therefore, we had to conclude that HS was masked by immunoreactants presumably containing nucleosomal antigens. More direct evidence for the involvement of nucleosomes in lupus nephritis is provided by studies showing both histones [86, 87] and DNA [88] in glomerular immune deposits in human and murine lupus nephritis. In one study the deposition of histones was almost exclusively found in albuminuric mice [87], underlining the role of histones and nucleosomes in the development of albuminuria in these mice. These *in vivo* data therefore support the concept that nucleosomal material may mediate antibody binding to the GBM for which HS is a ligand

In conclusion, both in human and murine lupus abnormalities in apoptosis have been described This aberrant apoptosis may contribute to lupus nephritis by mechanisms that have been summarized in Figure 3. Binding of nucleosome containing complexes to other basement membranes might lead to other organ manifestations of the disease. However, many questions remain to be answered. Future studies have to clarify the exact contribution of disturbed apoptosis in SLE. In addition studies are needed to identify the precise nature of the epitopes within the nucleosome recognized by auto-reactive T cells. Furthermore, the possibility should be studied that different epitopes within the nucleosome

dictate the production of different autoantibodies (such as anti-histone, anti-DNA, anti-nucleosome). Finally the exact composition of the nucleosome and the nucleosome/autoantibody complex determining affinity for the GBM should be clarified.

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Note added in proof

After submission of this review, two reviews have appeared about disturbed apoptosis in the *lpr* model (SINGER GG, CARRERA AC, MARSHAK-ROTHSTEIN A, MARTINEZ-A C, ABBAS AK. Apoptosis, Fas and systemic autoimmunity The MRL-*lpr/lpr* model *Curr Opin Immunol* 6 913–920, 1994, and NAGATA S, SUDA T: Fas and Fas ligand: *lpr* and *gld* mutations *Immunol Today* 16·39–43, 1995)

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CHAPTER 10

Summary.

Antibodies against double stranded DNA (dsDNA) are a serological marker for the autoimmune disease systemic lupus erythematosus (SLE). They have always been thought to play an important role in the etiopathogenesis of many organ manifestations of the disease. Especially nephritis, one of the most serious complications of SLE, has in the past been linked to the occurrence of anti-dsDNA antibodies. Classically, it was thought that DNA/anti-dsDNA complexes are trapped passively in the glomerular basement membrane (GBM), leading to local inflammation.
We challenged this concept and showed in previous work, described in ***chapter two***, that anti-dsDNA antibodies may cross-react with heparan sulfate (HS), which is an intrinsic constituent of the GBM and responsible for its negative charge. This cross-reactivity is not a property of the antibodies themselves but is based on histones and DNA (nucleosomes) bound to the antibodies. In our concept nucleosomes bridge the binding of antibodies to the anionic HS via the cationic histones within the nucleosome.

In ***chapter three*** in a prospective analysis in 72 consecutive SLE patients the correlation between plasma anti-HS reactivity and the occurrence of exacerbations with renal manifestations was studied. From these 72 patients 22 experienced 40 exacerbations. In 20 of these exacerbations renal symptoms were present. During exacerbations with renal manifestations both anti-HS and anti-dsDNA titers were higher than in exacerbations without renal symptoms. Anti-HS titers correlated significantly with anti-dsDNA antibody titers ($r_s = 0.57$; $p<0.05$). In some patients a high anti-dsDNA titer without anti-HS reactivity was detected, whereas anti-HS reactivity without anti-dsDNA reactivity was never found. These findings suggest that anti-HS reactivity is dependent of the presence of anti-dsDNA antibodies, whereas not all anti-dsDNA antibodies are complexed to nucleosomes and thus exhibit anti-HS reactivity.

In ***chapter four*** complexed monoclonal anti-nuclear antibodies, showing anti-HS reactivity, were prepared using various purification procedures. In this way antibody preparations containing nucleosomal material and pure non-complexed antibodies were obtained. Pure and complexed antibodies were tested *in vitro* for antigen specificity and were perfused into a rat kidney to study glomerular binding. In ELISA pure antibodies could not bind to HS (as expected) but surprisingly also DNA reactivity was lost after removal of nucleosomal material. In purified form only the

intact nucleosome was recognized. When the antibodies were complexed to nucleosomes they bound to DNA (both in ELISA and Farr assay), to HS and to histones.
When pure non-complexed antibodies were perfused into a rat kidney no binding was observed, whereas after perfusion of antibodies complexed to nucleosomal material binding of antibodies through the whole width of the GBM was observed. Histones could be detected in these GBM deposits and when after the renal perfusion, the blood circulation of the rat was restored for 15 minutes, deposition of rat complement was detected. In order to show that HS in the GBM was the ligand for the observed binding, HS was removed by renal perfusion of the HS degrading enzyme heparinase. When complexed antibodies were perfused after removal of HS by this procedure, binding was considerably reduced, showing that HS is an important but not the only ligand for the complexed antibodies in the GBM.

Since in chapter four it was shown that anti-nucleosome antibodies complexed to nucleosomes were able to bind to the GBM and initiate inflammation, in ***chapter five*** we studied whether histones and nucleosomes could actually be detected in biopsies of patients with lupus nephritis. We also studied whether the presence of nucleosomes and histones in these biopsies correlated with loss of HS staining in the GBM. This was done since in a recent study from our laboratories we found that HS staining was decreased in albuminuric MRL/l and NZB/W F1 lupus mice, while glomerular HS content was intact, suggesting that HS was masked by immune deposits. We studied kidney biopsies of 11 lupus patients with diffuse proliferative SLE glomerulonephritis (DPGN, WHO class IV) and 6 patients with membranous SLE glomerulonephritis (MGN, WHO class V). Sections of all biopsies were stained for the presence of histones, nucleosomes, DNA and IgG in the immune deposits and for the presence of HS and heparan sulfate proteoglycan (HSPG) core protein (which is the glycoprotein to which HS is linked) in the GBM. Histones were present in all patients with DPGN and in two out of six patients with MGN ($p<0.02$, Fisher exact test). Nucleosomes were detected in five patients with DPGN and in none of the MGN patients. HS staining was nearly absent in DPGN and only moderately reduced in MGN. A significant correlation between presence of histones and decrease of HS staining was found ($r_s = -0.77$). Staining of HSPG core protein was normal in all biopies. It is at present unclear whether the nucleosomal particle in the glomerular deposits consists mainly of histones or whether *in vivo* binding of anti-dsDNA and/or anti-nucleosome antibodies leads to masking of nucleosome specific epitopes.

In ***chapter six*** another possible pathogenic mechanism of anti-nucleosome antibodies is described. When the hybridomas producing monoclonal anti-nucleosome antibodies (mAbs) are inoculated intraperitoneally in mice, in immunofluorescence (IF) not only glomerular binding is observed but also binding to cell nuclei is found. This phenomenon is called *in vivo* anti-nuclear activity (*in vivo* ANA). Recently, it was described that some anti-DNA antibodies are able to bind to the cell surface, subsequently are internalized in the cytoplasm and thereafter are transported to the nucleus (*in vivo* ANA), which leads to pathological consequences like decreased cell proliferation, increased protein synthesis, glomerular hypercellularity and proteinuria. We could show that when antibodies complexed to nucleosomes are injected intravenously in naive mice, after 45 minutes *in vivo* ANA is observed, whereas after injection of pure non-complexed antibodies hardly any nuclear binding is seen. In additional experiments using well defined human cell lines, cell surface binding of antibodies also appeared to be a property of anti-nucleosome antibodies complexed to nucleosomes, whereas pure non-complexed antibodies did not bind. However, when complexed antibodies were incubated up to 24 hours with living cells, only internalization of antibodies in cytoplasmic vesicles was found and no nuclear binding. We hypothesized that *in vivo* ANA observed in IF may be related to the fixation procedure. To study this, from mice bearing the hybridoma producing the anti-nucleosome mAbs intraperitoneally, a small part of the kidney was processed for standard IF using acetone fixation after cutting of the sections, whereas the remaining part was *in vivo* fixed with a mixture of 0.01 M sodium periodate, 0.075 M lysine HCl, 0.0375 M Na_2HPO_4 and 2% paraformaldehyde (PLP) and studied in both IF and IEM. After standard IF, as anticipated, *in vivo* ANA was observed, whereas after *in vivo* fixation with PLP only localization of antibodies in cytoplasmic vesicles and no nuclear binding was found. From these experiments we conclude that anti-nucleosome antibodies complexed to nucleosomes can bind to the cell surface and are subsequently internalized. *In vivo* ANA however, is a fixation related phenomenon.

Until now little is known about the characteristics of the epitopes on the intact nucleosome which are recognized by anti-nucleosome antibodies. In ***chapter seven*** a panel of six anti-nucleosome mAbs derived from lupus mice was characterized in ELISA on nucleosomes and subnucleosome structures (H2A/H2B dimer, H3/H4 tetramer, H2A/H2B-DNA complex and H3/H4-DNA complex) and on a panel of 53 overlapping synthetic histone peptides covering the entire sequence of the five

histones. By definition these antibodies showed no or low reactivity towards DNA or the individual histones compared to their reactivity towards nucleosomes. In the histone peptide ELISA we found that only two mAbs recognized one of the 53 peptides. Since the reactivity found hardly exceeded background levels, we concluded that linear histone epitopes are not important for the binding of these anti-nucleosome antibodies. In the nucleosome and subnucleosome ELISAs all mAbs showed different reactivity patterns. Three mAbs showed higher reactivity towards the intact nucleosome than to subnucleosome structures. For two of them the primary nucleosomal epitope was located on H2A/H2B-DNA, whereas for the other mAb this primary epitope was located on H3/H4/DNA. From the remaining three, two mAbs were directed against an epitope on H2A/H2B-DNA and one mAb recognized an epitope on H3/H4-DNA. In the subnucleosome ELISA all but one recognized more than one epitope. When DNA was coated with a variety of cationic molecules for all mAbs one specific nucleosomal epitope could be identified, whereas the reactivity with other subnucleosomes was comparable to the reactivity towards DNA coated with these cationic molecules. In inhibition experiments both in ELISA and in immunofluorescence (IF) it was found that only one of the mAbs recognizing an epitope on H2A/H2B-DNA as primary epitope could be inhibited by H2A/H2B-DNA in fluid phase. The two mAbs recognizing an epitope on H3/H4-DNA as primary epitope could be inhibited by H3/H4-DNA in fluid phase. From these experiments we conclude that some epitopes on H2A/H2B-DNA recognized by certain anti-nucleosome mAbs are not expressed by H2A/H2B-DNA in fluid phase.
These mAbs all recognize different nucleosomal epitopes on both H2A/H2B-DNA and H3/H4-DNA. Our findings support the view that in SLE the whole nucleosome is the auto-antigen which triggers the formation of anti-nucleosome antibodies.

It is now generally accepted that the auto-immune response in SLE is antigen driven and T-cell dependent. The nucleosome has been proposed as an important immunogen leading to the formation of not only anti-nucleosome, but also anti-dsDNA and anti-histone antibodies. In this view a nucleosomal epitope is involved in the formation of anti-dsDNA antibodies. We therefore hypothesized that anti-dsDNA antibodies might recognize more than DNA alone and tested in ***chapter eight*** 6 monoclonal anti-dsDNA antibodies derived from lupus mice on the panel of 53 overlapping synthetic histone peptides described above. We found that four out of six mAbs recognized one and the same histone peptide, i.e. the H3 83-100 peptide. Both DNA and H3 83-100 were able to inhibit the binding of these mAbs to DNA and H3

83-100. We hypothesize that this peptide, together with DNA forms a nucleosomal epitope, which evokes the formation of auto-antibodies capable of binding to DNA and H3 83-100 separately. It has recently been postulated that the nucleosome is processed by antigen presenting cells and that cryptic nucleosomal peptides are presented to T-cells, which help B-cells in the production of anti-dsDNA antibodies. Maybe H3 83-100 is one of these cryptic epitopes.

Since the most likely source of nucleosomes in the circulation is from apoptotic cells and disturbances in apoptosis have been described in both murine and human lupus, in ***chapter nine*** the literature concerning apoptosis, nucleosomes and nephritis in SLE is reviewed. Disturbances in apoptosis can lead to both the persistance of autoreactive T cells and to release of nucleosomes which may be altered (and become more immunogenic). Decreased phagocytosis, which has been described in SLE, may lead to prolonged survival of nucleosomes in the circulation. These altered nucleosomes than may be a target for autoreactive T-cells, which leads to the production of anti-nucleosome, anti-dsDNA and anti-histone antibodies. As already described in chapters 2 and 3, nucleosomes may mediate the binding of these antibodies to the GBM. So the nucleosome seems to play a central role in SLE, both in the induction and in the effector phase of the disease.

CHAPTER 11

Samenvatting.

Antistoffen tegen dubbelstrengs DNA (dsDNA) zijn kenmerkend voor de autoimmuun ziekte systemische lupus erythematosus (SLE). Men is er altijd van uitgegaan dat deze antistoffen een belangrijke rol spelen in de ontstaanswijze van vele verschijnselen van deze ziekte. Met name voor het optreden van nefritis, één van de ernstigste complicaties van SLE, is een verband met anti-dsDNA antistoffen beschreven. Van oudsher is er gedacht dat DNA/anti-dsDNA complexen vastlopen in de glomerulaire basaal membraan (GBM), hetgeen tot een locale ontstekingsreactie zou leiden.
Wij vroegen ons af of deze zienswijze juist was en toonden in vroeger werk aan, (beschreven in ***hoofdstuk twee***) dat anti-dsDNA antistoffen kunnen kruisreageren met heparaan sulfaat (HS). HS is een onderdeel van de GBM en verantwoordelijk voor diens negatieve lading. Deze kruisreactiviteit wordt niet door de antistoffen zelf veroorzaakt, maar door histonen en DNA (nucleosomen) gebonden aan de antistoffen. In onze visie vormen nucleosomen een brug voor de binding van antistoffen aan het negatief geladen HS in de GBM, waarbij de positief geladen histonen in het nucleosoom aan HS binden.

In ***hoofdstuk drie*** werd in een prospectieve analyse bij 72 SLE patiënten de correlatie tussen plasma anti-HS reactiviteit en het optreden van exacerbaties met renale verschijnselen bestudeerd. Van deze 72 patiënten kregen er 22 gezamenlijk 40 exacerbaties. Bij 20 van deze exacerbaties waren er onder andere renale symptomen. Gedurende exacerbaties met renale verschijnselen waren zowel anti-HS als anti-dsDNA hoger dan in exacerbaties zonder renale symptomen. De anti-HS titers toonden een significante correlatie met anti-dsDNA antistof titers ($r_s = 0,57$; $p<0,05$). Bij sommige patiënten werd een hoge anti-dsDNA titer zonder anti-HS gevonden, terwijl anti-HS reactiviteit zonder anti-dsDNA reactiviteit niet gezien werd. Deze bevindingen maken het aannemelijk dat anti-HS reactiviteit afhankelijk is van de aanwezigheid van anti-dsDNA antistoffen, terwijl niet alle anti-dsDNA antistoffen gecomplexeerd zijn aan nucleosomen en zo anti-HS reactiviteit vertonen.

In ***hoofdstuk vier*** werden 3 anti-nucleaire monoclonale antistoffen die in kweek supernatant anti-HS reactiviteit vertoonden, op verschillende manieren opgezuiverd. Op deze wijze werden antistoffen gecomplexeerd aan nucleosomaal materiaal en zuivere niet-gecomplexeerde antistoffen verkregen. Van deze zuivere en gecomplexeerde antistoffen werd *in vitro* de antigene specificiteit bepaald. Daarnaast werden rattenieren geperfundeerd met deze antistof preparaten om zo de binding in

de glomerulus te bestuderen. In ELISA bleken zuivere antistoffen niet aan HS te binden (hetgeen ook voorspeld was), maar onverwachts ging ook de DNA reactiviteit verloren na verwijdering van het gecomplexeerde nucleosomale materiaal. In zuivere toestand herkenden deze antistoffen slechts het intacte nucleosoom. Alleen als de antistoffen gecomplexeerd waren aan nucleosomen konden ze aan DNA (in ELISA en Farr assay), HS en histonen binden.
Na renale perfusie van zuivere niet-gecomplexeerde antistoffen werd geen binding gezien, terwijl na perfusie van antistoffen gecomplexeerd aan nucleosomaal materiaal binding van antistoffen door de gehele breedte van de GBM gevonden werd. Histonen werden in deze GBM deposities aangetoond. Als na renal perfusie de circulatie van de rat gedurende 15 minuten hersteld werd, werd depositie van rat complement waargenomen. Teneinde aan te tonen dat HS in de GBM de ligand voor de binding van deze gecomplexeerde antistoffen was, werd HS van de GBM verwijderd door renale perfusie van het enzym heparinase, hetgeen in staat is HS af te breken. Als na deze procedure gecomplexeerde antistoffen werden geperfundeerd, bleek de binding van antistoffen aan de GBM aanzienlijk verminderd.
Uit deze proeven concluderen we dat anti-nucleosoom antistoffen gecomplexeerd aan nucleosomaal materiaal kunnen binden aan de GBM en dat HS een belangrijke, doch niet de enige ligand in de GBM is waaraan deze gecomplexeerde antistoffen binden.

Daar in hoofdstuk 4 aangetoond was dat anti-nucleosoom antistoffen gecomplexeerd aan nucleosomen aan de GBM konden binden en ontsteking konden veroorzaken, analyseerden we in ***hoofdstuk vijf*** of histonen en nucleosomen ook daadwerkelijk in nierbiopsieën van patienten met lupus nefritis aantoonbaar waren. Bovendien bestudeerden we of de aanwezigheid van nucleosomen en histonen correleerde met verlies van HS kleuring in de GBM. In een recente studie hebben we namelijk gevonden dat de HS kleuring is afgenomen in MRL/l muizen met een eiwitlek, terwijl het glomerulaire HS gehalte niet veranderd is, hetgeen doet vermoeden dat HS is afgedekt door immuun deposities. We bestudeerden nierbiopsieën van 11 lupus patiënten met diffuus proliferatieve glomerulonefritis (DPGN, WHO klasse IV) en 6 patiënten met membraneuze glomerulonefritis (MGN, WHO klasse V). Coupes van alle nierbiopsieën werden gekleurd op de aanwezigheid van histonen, nucleosomen, DNA en IgG in de immuun deposities. Bovendien werd de kleurbaarheid van HS en eiwitdeel van heparaan sulfaat proteoglycaan (HSPG) (het glycoproteine waarvan HS een zijketen is) in de GBM getest. Histonen waren aanwezig in de glomerulaire deposities van alle patiënten met DPGN en in 2 van de 6 patiënten met MGN

($p<0,02$; Fisher exact test). Nucleosomen werden aangetoond in de biopsieën van 5 patiënten met DPGN en in geen van de MGN patiënten. De HS kleuring was vrijwel verdwenen in de GBM van DPGN patiënten, terwijl deze slechts in lichte mate verminderd was bij MGN patiënten. Er was een significante correlatie tussen de aanwezigheid van histonen en de afname van HS kleuring (r_s = -0,77). De kleurbaarheid van het eiwitdeel van HSPG was normaal in alle biopsieën. Het is onduidelijk of het nucleosomale partikel wat aanwezig is in glomerulaire immuun deposities voornamelijk uit histonen bestaat, of dat *in vivo* binding van anti-dsDNA en/of anti-nucleosoom antistoffen bepaalde nucleosomale epitopen afdekt.

In ***hoofdstuk zes*** wordt een ander mechanisme beschreven waardoor anti-nucleosoom antistoffen pathogeen kunnen zijn. Als hybridomen die monoclonale anti-nucleosoom antistoffen produceren intraperitoneaal bij muizen worden geplaatst, wordt met immunofluorescentie (IF) niet alleen glomerulaire binding gezien maar ook binding van antistoffen aan de cel kernen. Dit fenomeen heet *in vivo* anti-nucleaire activiteit (*in vivo* ANA). Onlangs werd beschreven dat bepaalde anti-dsDNA antistoffen aan het cel oppervlak kunnen binden, vervolgens in het cytoplasma opgenomen worden en daarna naar de celkern getransporteerd worden (*in vivo* ANA), hetgeen pathogeen zou zijn en zou leiden tot verminderde cel proliferatie, toegenomen eiwit synthese, glomerulaire hypercellulariteit en proteinurie.
Als wij antistoffen gecomplexeerd aan nucleosomen intraveneus aan normale muizen toedienden, werd na 45 minuten *in vivo* ANA gezien, terwijl injectie van zuivere niet-gecomplexeerde antistoffen niet tot binding van antistoffen leidde. Vervolgens bleek in experimenten met humane cellijnen dat ook binding aan het cel oppervlak een eigenschap van antistoffen gecomplexeerd aan nucleosomen was, terwijl niet-gecomplexeerde antistoffen niet bonden. Echter, incubatie van levende cellen met gecomplexeerde antistoffen gedurende 24 uur leidde slechts tot opname van antistoffen in cytoplasmatische vesikels en niet tot binding aan de kern. Wij veronderstelden dat *in vivo* ANA zoals die in IF gezien werd, wellicht samenhing met de methode van fixeren. Om dit aan te tonen, werd van muizen bij wie het hybridoom wat de anti-nucleosoom antistoffen produceerde intraperitoneaal geplaatst was, een klein deel van de nier genomen en verwerkt voor standaard IF met aceton fixatie na het snijden van coupes. Een ander deel van dezelfde nier werd *in vivo* gefixeerd met een oplossing van 0,01 M perjodaat, 0,075 M lysine HCl, 0,0375 M Na_2HPO_4 en 2% paraformaldedyde (PLP) en bestudeerd in zowel IF als immuno electronen microscopie. Na standaard IF werd, zoals te verwachten was, *in vivo* ANA

gezien, terwijl na *in vivo* fixatie met PLP geen kernbinding, maar slechts opname in cytoplasmatische vesikels gevonden werd. Uit deze experimenten concluderen we dat anti-nucleosoom antistoffen gecomplexeerd aan nucleosomen aan het cel oppervlak kunnen binden en dat de antistoffen vervolgens in het cytoplasma worden opgenomen. Het optreden van *in vivo* ANA echter is afhankelijk van de fixatie methode.

Op dit moment is er betrekkelijk weinig bekend over de epitopen op het nucleosoom welke door anti-nucleosoom antistoffen herkend worden. In ***hoofdstuk zeven*** wordt een panel van 6 anti-nucleosoom antistoffen welke zijn verkregen uit lupus muizen, gekarakteriseerd in ELISA door ze te testen op nucleosomen en subnucleosomale structuren (H2A/H2B dimeer, H3/H4 tetrameer, H2A/H2B-DNA complex en H3/H4-DNA complex) en op een panel van 53 overlappende synthetische histon peptiden die de volledige aminozuur sequentie van de 5 histonen omvatten. Per definitie hadden deze antistoffen geen of een lage reactiviteit met DNA of de individuele histonen ten opzichte van de reactiviteit met het intacte nucleosoom. In de histon peptide ELISA vonden we dat slechts 2 monoclonale antistoffen één van de 53 peptiden herkenden. Deze reactiviteit kwam nauwelijks boven de achtergrond uit, waaruit we concludeerden dat lineaire histon epitopen van ondergeschikt belang zijn voor de binding van deze anti-nucleosomale antistoffen. In de nucleosoom en subnucleosoom ELISAs vonden we dat alle 6 antistoffen verschillend reageerden. Drie antistoffen toonden een hogere reactiviteit met het intacte nucleosoom dan met subnucleosomale structuren. Voor twee van hen bevond het primaire nucleosomale epitoop zich op H2A/H2B-DNA en voor de derde op H3/H4-DNA. Twee van de overige antistoffen waren gericht tegen een epitoop op H2A/H2B-DNA en de laatste tegen een epitoop op H3/H4-DNA. In de subnucleosoom ELISA herkenden alle antistoffen op één na meer dan één epitoop. Wanneer DNA met verschillende cationische moleculen gecoat werd, bleek voor alle antistoffen één specifiek nucleosomaal epitoop te identificeren te zijn, terwijl de reactiviteit met het andere subnucleosoom vergelijkbaar was met de reactiviteit met DNA gecoat met willekeurige cationische moleculen. In inhibitie experimenten werd zowel in ELISA als in IF gevonden dat slechts één antistof die een epitoop op H2A/H2B-DNA herkende, geremd kon worden door H2A/H2B-DNA in vloeibare fase. De twee antistoffen die een epitoop op H3/H4-DNA herkenden konden beiden wel door H3/H4-DNA in vloeibare fase geremd worden. Uit deze experimenten concluderen we dat sommige epitopen op H2A/H2B-DNA die door bepaalde anti-nucleosomale antistoffen in ELISA herkend worden, niet op H2A/H2B-

DNA in vloeibare fase voorkomen.
Deze 6 monoclonale antistoffen herkennen allen verschillende epitopen op zowel H2A/H2B-DNA en H3/H4-DNA. Deze bevindigen bevestigen de veronderstelling dat in SLE het hele nucleosoom het auto-antigen is dat de vorming van deze anti-nucleosoom antistoffen veroorzaakt.

Op dit moment wordt verondersteld dat de auto-immuun reactie in SLE antigeen en T-cel afhankelijk is. Het nucleosoom zou een belangrijk immunogeen zijn, hetgeen uiteindelijk leidt tot de vorming van niet alleen anti-nucleosoom, maar ook anti-dsDNA en anti-histon antistoffen. Volgens deze visie is een nucleosomaal epitoop betrokken bij de vorming van anti-dsDNA antistoffen. Daarom veronderstelden we dat anti-dsDNA wellicht meer dan DNA alleen herkennen en testten we in ***hoofdstuk acht*** 6 monoclonale antistoffen welke waren verkregen van lupus muizen op het panel van 53 overlappende synthetische histon peptiden, zoals dat in hoofdstuk 7 beschreven is. Wij vonden dat 4 van deze 6 antistoffen één en hetzelfde histon peptide herkenden namelijk het 83-100 peptide van histon H3. Zowel DNA als H3 83-100 waren in staat om de binding van deze antistoffen aan DNA en H3 83-100 en vice versa te remmen. We hypothetiseren dat dit peptide samen met DNA een nucleosomaal epitoop vormt, waartegen antistoffen ontstaan, die zowel DNA als H3 83-100 apart kunnen binden. Recent is gepostuleerd dat het nucleosoom wordt verwerkt door antigeen presenterende cellen en dat cryptische nucleosomale peptiden aan de T-cellen aangeboden worden, welke de B-cellen bijstaan bij de productie van anti-dsDNA antistoffen. Wellicht is H3 83-100 één van deze cryptische epitopen.

Daar apoptotische cellen de meest voor de hand liggende bron voor nucleosomen in de circulatie zijn en verstoring van apoptose zowel bij mensen als bij muizen beschreven is in SLE, hebben we in ***hoofdstuk negen*** de literatuur betreffende apoptose, nucleosomen en nefritis in SLE samengevat. Verstoring van apoptose kan zowel tot langdurige overleving van autoreactieve T-cellen leiden, als tot het vrijkomen van nucleosomen die wellicht veranderd en zo immunogeen zijn. Verminderde fagocytose, hetgeen ook bij SLE beschreven is, kan leiden tot een verlengde overleving van nucleosomen in de circulatie. Deze veranderde nucleosomen kunnen het doelwit zijn van autoreactieve T-cellen, hetgeen leidt tot de vorming van anti-nucleosoom, anti-dsDNA en anti-histon antistoffen. Zoals beschreven in de hoofdstukken 2 en 3, kunnen nucleosomen de binding van deze antistoffen aan de GBM bewerkstelligen. Aldus lijken nucleosomen een centrale rol in SLE te spelen, zowel bij de inductie als in de effector-fase van de ziekte.

DANKWOORD

"Ben je handig?" vroeg Jo begin 1991 aan me. Ik begreep terstond dat een al te eerlijk antwoord mijn carrière geen goed zou doen en heb iets filosofisch geantwoord, in de trand van wat is handig of zoiets.
Aan Jo heb ik zeer veel te danken. Vele van de in dit proefschrift beschreven proeven vonden hun oorsprong in zijn geest. Daarnaast corrigeerde hij de stukken zeer nauwgezet en gaf manuscripten daardoor een enorme meerwaarde.
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En last but not least Yvonne, die mij steunde, motiveerde, aanspoorde en zorgt dat er in mijn chaos nu en dan wat lijn gebracht wordt.

CURRICULUM VITAE

Kees Kramers werd geboren op 6 januari 1962 te Dirksland. In 1979 behaalde hij het einddiploma Atheneum-B aan de Rijksscholengemeenschap Goeree Overflakkee te Middelharnis. In datzelfde jaar ving hij aan met de studie medicijnen aan de Rijks Universiteit te Leiden. Van 1982-1984 was hij student-assistent op de afdeling Heelkunde, Academisch Ziekenhuis Leiden, alwaar onderzoek verricht werd naar resultaten van operatief ingrijpen vanwege obliferatief vaatlijden in het aorto-iliacale traject. Van 1982 tot 1983 was hij secretaris van de studentenroeivereniging "Asopos de Vliet". In 1986 werd de studie medicijnen succesvol afgerond en werkte hij gedurende ruim een jaar als arts-assistent op de afdeling Interne Geneeskunde van het Medisch Centrum Alkmaar. In 1987 startte hij de opleiding tot internist in het Sint Maartens Gasthuis te Venlo (opleider Dr J.J.J. Mattousch), welke in 1989 werd voortgezet in het Sint Radboud Ziekenhuis te Nijmegen (opleider Prof. Dr A. van 't Laar). Van 1991 tot 1994 werd de opleiding onderbroken vanwege het verrichten van onderzoek naar de ontstekingsreactie van lupus nefritis, hetgeen resulteerde in dit proefschrift (werkgroepleider Prof. Dr J.H.M. Berden). In 1994 werd de opleiding tot internist hervat (opleider inmiddels Prof. Dr J.W.M. van der Meer).
Hij is sedert 1990 getrouwd met Yvonne Heijdra en vader van Bart en Tim.

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STELLINGEN

behorende bij het proefschrift

Anti-nucleosome auto-antibodies

in systemic lupus erythematosus

Kees Kramers

12-12-1995

1. Nucleosomaal materiaal kan de binding van anti-nucleosoom antistoffen aan de GBM mediëren en speelt op deze manier een rol in de initiatie van lupus nefritis.
(dit proefschrift)

2. Anti-nucleosoom antistoffen, zoals die bij SLE worden aangetroffen, zijn zeer heterogeen en herkennen verschillende nucleosomale epitopen.
(dit proefschrift)

3. Het feit dat in glomerulaire deposities van lupus patienten histonen en nucleosomen aantoonbaar zijn, terwijl DNA niet aankleurt, suggereert dat DNA is afgedekt door antistoffen.
(dit proefschrift)

4. Het feit dat sommige anti-DNA antistoffen een histon peptide herkennen, suggereert dat nucleosomen van belang zijn voor de vorming van deze antistoffen.
(dit proefschrift, Mohan et al J.Exp.Med. 1993;177:1367)

5. Apoptose speelt een belangrijke rol in de pathogenese van SLE, zowel in de initiatie als in de effector fase van de ziekte.

6. Het is tot op heden onmogelijk alle antistoffen uit plasma of glomerulaire eluaten op te zuiveren zonder selectief reactiviteit te verliezen.

7. De polyreactiviteit van glomerulaire eluaten zoals beschreven door Sabbaga et al (J.Autoimmunity 1990;3:215) is ongetwijfeld veroorzaakt door antistoffen gecomplexeerd aan nucleosomaal materiaal.

8. De bestudering van anti-nucleosomale antistoffen in SLE en drug-induced lupus zal inzicht kunnen verschaffen over de eigenschappen die de nefritogeniciteit bepalen.

9. Bij de selectie voor geschikte kandidaten voor de opleiding tot internist zou een meting van het kukel moeten geschieden.
(vrij naar Marten Toonder)

10. Voor de zuiverheid van beoordeling van onderzoek door paraklinische specialismen zoals radiodiagnostiek en pathologie zou vermelding van klinische gegevens achterwege moeten blijven.

11. Een goede onderzoeker heeft een goede analist.

12. Door condooms en geboortebeperking te verbieden, werkt het Vaticaan mee aan een catastrofe in Afrika.

13. Principiële tegenstanders van actieve euthanasie begeleiden geen patiënten.

14. Het is met moraal, zoals met invoegen in de file. Degene die eerder invoegt is een sukkel, degene die later invoegt een schoft.

15. A small step for a man, a giant leap for mankind.
(dit proefschrift)